Handbook of Basic Microtechnique

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of
Basic Microtechnique

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of
Basic Microtechnique

by

PETER GRAY, Ph.D., D.I.C.
Head, Department of Biological Sciences
University of Pittsburgh

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Preface

This little book is intended as a useful first aid to the student or technical worker faced with the problem of preparing animal or plant material for microscopical examination. In cytology, histology, and pathology—microtechnical work generally—there are certain basic procedures to be followed and the professor or research worker may not be available for consultation on the more elemental steps. At the same time a reference work may require much reading of theory before such an essential working step is made clear. This handbook tries to obviate the difficulty and refresh the memory on a particular point, thereby saving time and effort.

The book, therefore, is intended to supplement Gray's *Microtechnique,* a larger reference work now in preparation. It seems to both the author and his publishers that the field of microscopical technique presents an unusual dilemma. One horn is the necessity of providing an authoritative and exhaustive account of the main techniques in use over the last half century. The other horn is the necessity of providing the college student and technician with an inexpensive book for quick consultation and answers, a useful tool shorn of reference qualities. To this end the author and publishers will present two books: the one, in preparation, a comprehensive reference and this one, a small, quickly usable handbook. Such a procedure avoids the customary compromise which is neither exhaustively complete nor inexpensive enough for a text.

This present work makes no pretension to scholarship in that it contains no reference to the literature of the subject—this is left to the larger work. The *Handbook of Basic Microtechnique* does aspire, however, to wide usefulness. It is hoped that it may be completely useful in itself, answering many questions for the thousands of workers in the various fields of microtechnical procedures.

Acknowledgment is made with thanks to the Fisher Scientific Company for figures 1, 2, 3, 4, 5, 6, 7, 8, 17, 21, 32, 33, 56, 57 and to the Arthur H. Thomas Company for figure 55.

THE AUTHOR

Contents

Preface v

Part 1

Materials and Processes of Slide-Making

1. Materials and Equipment 3

Slides and Coverslips — Containers for Handling Objects — Equipment for Handling Slides.

2. Fixation and Fixatives 6

The Nature of the Process—Dichromate and Chromate Fixatives—Mercuric Fixatives—Picric Acid Fixatives—Fixatives of Kolmer and Petrunkewitsch—Removal of Fixatives—Softening Hard Objects—Narcotization.

3. Stains and Staining 15

The Nature of the Process—Direct Nuclear in Hematoxylin—Indirect Hematoxylin Staining—Indirect Carmine Nuclear Stains—Synthetic Nuclear and Bacterial Stains—Simple Contrast Stains—Double Contrast Stains—Complex Zoological Staining Techniques—Complex Botanical Staining Techniques—Stains for Special Purposes.

4. Dehydrating and Clearing 30

The Nature of the Process—Alcohol Series—Dioxane and Cellosolve—Essential Oils for Clearing — Benzol, Toluol, and Xylol — Necessity for Perfect Dehydration.

5. Mounts and Mountants 35

Gum Media—Resinous Media.

6. Making Wholemounts 39

Slides for Wholemounts—Cavity Slides—Cells—Mounting Directly in Gum and Resinous Media—Narcotization and Killing—Dehydration and Clearing—Mounting in Balsam.

7. Making Smears 49

Cleaning Slides—Smearing—Fixing and Staining Smears.

8. Making Sections 52

The Nature of the Process—Free Hand Sections—Paraffin and Sections—Dehydrating Tissues—Clearing Tissues—Choice of an Embedding Medium—Process of Embedding—Preparing the Block—Cutting Sections—Mounting Sections on Slides—Staining and Mounting Sections—Frozen Sections.

9. Cleaning, Labeling, and Storing Slides 75

Cleaning and Finishing Gum Mounts—Cleaning and Finishing Resin Mounts—Labels and Labeling—Slide Boxes and Cabinets.

Part 2

Specific Examples of Slide-Making

1. Preparation of a Wholemount of the Mite in the Medium of Berlese...... 81
2. Preparation of a Wholemount of Pectinatella in Balsam after Staining in Grenacher's Alcoholic Borax Carmine 84
3. Preparation of a Wholemount of a Chicken Embryo in Balsam after Staining in Carazzi's Alum Hematoxylin 89
4. Preparation of a Wholemount of the Liver Fluke after Staining in Mayer's Carmalum 95
5. Preparation of a Smear to Show Monocystis in the Seminal Fluid of an Earthworm Stained in Ehrlich's Acid Alum Hematoxylin........ 98
6. Preparation of a Bacterial Smear Stained by the Technique of Lillie....... 100
7. Preparation of a Bacterial Film to Demonstrate Gram Positive Organisms.. 102
8. Demonstration of Tubercle Bacilli in Sputum by the Ziehl-Neelsen Technique 104
9. Preparation of a Free-hand Section of a Root Stained by the Acid Fuchsin-iodine Green Stain of Chamberlain 106
10. Preparation of a Section of the Intestine of the Frog Stained in Celestin Blue B-eosin........ 110
11. Preparation of a Transverse Section of a Stem of Aristolochia Stained by the Method of Johansen........ 117
12. Demonstration of Spermatogenesis in the Testes of the Rat Using Allen's Fixative and Iron Hematoxylin 120
13. Preparation of a Paraffin Section of the Tongue of a Rat Stained with Celestin Blue B and van Gieson's Picro-fuchsin........ 125
14. Preparation of a Paraffin Section of an Amphioxus Using Mallory's Triple Stain 128
15. Preparation of a Series of Demonstration Slides Each Having Six Typical Transverse Sections of a 72-hour Chick Embryo Stained with Ehrlich's Acid Alum Hematoxylin........ 131

Index 135

Part 1

Materials and Processes of Slide-Making

1

Materials and Equipment

This brief introductory chapter is intended for those who not only have never made a microscope slide but also have never even watched one being made, so that they are unacquainted with the names of any of the pieces of equipment used in its production. As these names will be used freely throughout the other chapters in this book, it is well at the present time by reference to illustration and descriptions to make sure that the beginning student knows what the terms mean.

Slides and Coverslips. The "microscope mount" consists essentially of something intended for examination under the microscope, which is held between a "slide" and "coverslip." The slide is almost invariably a piece of thin glass 3 in. by 1 in., though on rare occasions big series of sections are mounted on 3- by 1½-in. or even 3- by 2-in. slides. It is a great mistake to use slides which are too thin, for not only are they very easily broken but they also do not work well with the condensers of most modern microscopes. A thickness of approximately 1 mm. is the optimum, but slides as thick as 1¼ mm. are perfectly satisfactory. It is essential that the surface of the slide be flat and that the glass of which it is composed be as stable as possible for it will have to be passed through a great number of reagents. These stable glass slides are usually called "noncorrosive," and it is a great pity to use any other kind.

Coverslips are circles, squares, or rectangles of thin glass; they are rarely, particularly in the larger sizes, completely flat. They are made in four thicknesses known as No. 0, No. 1, No. 2, and No. 3, each of which has its own special use. No. 0 coverslips, which are about 0.09 mm. thick, are very difficult to handle and should be used only on preparations which are to be examined with an oil-immersion objective. They are rarely used today since most people, even for immersion lenses, prefer a No. 1 coverslip, which averages about 0.15 mm. in thickness. No. 1 coverslips are easier to handle and to clean than are No. 0. No. 2 coverslips, which average about 0.20 mm. in thickness, are generally used on wholemounts, which are not customarily examined with the highest powers of the microscope. They are sufficiently thick to be easy to clean without breakage and sufficiently thin to be used with anything except an oil-immersion objective. No. 3 coverslips, averaging about 0.30 to 0.35 mm. in thickness, are used only in making covers for dry wholemounts, which are not described in this book. They should never be used for any other purpose.

Circular coverslips are available in sizes from ⅜ to ⅞ in. in diameter, and the size most commonly employed is a ¾ in. (18 mm.). For class purposes a

½-in. size is much more convenient and is quite large enough for the majority of wholemounts. Square coverslips come in the same sizes as do the circular ones. The choice between circular and square depends entirely on the preference of the mounter. Square coverslips are a trifle easier to handle, but round coverslips have the advantage that one can "ring" the slide if one is making fluid or dry mounts. These types of mounts, however, are not described in this book, for they are unsuitable for elementary students. Rectangular coverslips intended for use on 3- by 1-in. slides are always 22 mm. across their narrowest dimension and may be obtained 30, 40, or 50 mm. in length. It is rarely wise to use a coverslip longer than 50 mm., for insufficient space will be left on the end of the slide for the application of the label. Coverslips intended for use with 1½-in. slides are usually 35 mm. on their shortest dimension, and those intended for use with 2-in. slides are generally 43 mm. on their shortest dimension. Coverslips for use with larger slides are almost invariably 50 mm. long.

Containers for Handling Objects. Objects intended for microscopical examination have to go through a variety of processes, which are described in the next few chapters. Either the object may be put through these processes and subsequently mounted or it may be attached to a slide and the slide put through the processes. In either case a special container is required. Small objects are usually transferred between solutions either with a pipette (Fig. 1) or a section lifter (Fig. 2), the choice between the two depending upon the size and shape of the object. Where the object will stay for only a short time in a solution, it is customary to employ syracuse watch glasses (Fig. 3). These are called watch glasses because they have replaced the more conventional type of watch glass in laboratory technique. They have the advantage over the conventional type of being less easy to upset, and are so shaped that they may be stacked one on top of the other, both for storage and prevention of too-rapid evaporation of the contained solutions. They would be far better called syracuse "dishes," but the name watch glass is still in customary use. Embryological watch glasses (Fig. 4) are used less for soaking objects in various solutions than for embedding objects in paraffin. They are very convenient for the latter purpose. They may also be used in place of syracuse watch glasses and have the advantage that they are provided with a cover which prevents evaporation of the contained solutions. They are not, however, so convenient as the conventional stender dish (Fig. 5), which is furnished with a round, ground-on cover. If this cover is touched with a little petrolatum, it is possible to keep alcohol for several days in the dish without loss. When an object has to be stored for any length of time in a fluid, it is much better to use a vial (Fig. 6), the type shown having a screw cap of plastic. These are more expensive than the old-

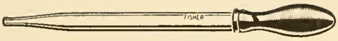

FIG. 1. Pipette.

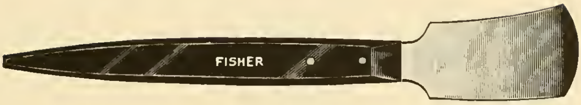

FIG. 2. Section lifter.

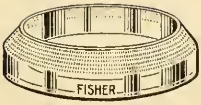

FIG. 3. Syracuse watch glass.

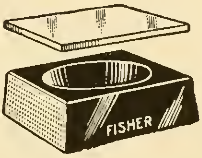

FIG. 4. Embryological watch glass.

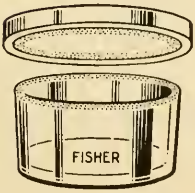

FIG. 5. Stender dish.

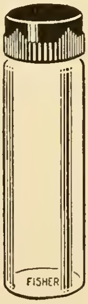

FIG. 6. Screw-cap vial.

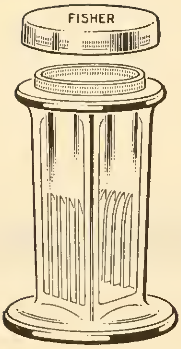

FIG. 7. Standard coplin jar.

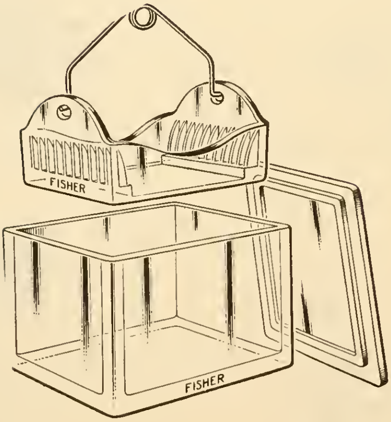
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FIG. 8. Rectangular slide jar.

fashioned corked vials but they are so much better that they are worth the additional expense. They should be available to the mounter in a great variety of sizes. Where a number of very small objects is to be stored, it is convenient to place each object in a little vial plugged with cotton and then to accumulate these vials in a larger screw-cap vial containing the preservative fluid. In this way, many hundreds of minute specimens may be kept separate and safe for long periods.

Equipment for Handling Slides. Where the object which is to be treated is attached to a slide, it is necessary to have special equipment to keep the slides separate as they are put through the solutions. If only a single slide is being handled, it is possible to use a vial, provided that the vial is more than 1 in. in diameter. Almost invariably, however, one handles several slides at one time, and for this purpose it is almost universal to use a coplin jar (Fig. 7). These rectangular jars are furnished with a series of grooves which hold a number of slides apart. Most coplin jars hold six slides, but it is possible to obtain them to hold as many as 12. The number of slides may be doubled by placing two slides back to back and sliding this sandwich into a groove, but this is very unsatisfactory because reagents diffuse so slowly from between the slides.

Coplin jars are made to handle only 3- by 1-in. slides. When one is dealing with the larger size, it is necessary to use a rectangular jar (Fig. 8) into which the slides are placed with their long edge downward. The jar shown contains a removable glass rack, so that a number of slides may be transferred from one jar to another without handling each one separately. This type is a great deal more expensive than the conventional rectangular jar with grooves down the side but is so much more convenient to use that it should be obtained where possible.

A set of eight jars, either coplin or of the rectangular type, is the minimum which is required for ordinary purposes of slide-making.

In addition to the items mentioned, there are a large number of specialized pieces of equipment, such as microtomes, warming tables, paraffin, and embedding ovens, which are part of the regular laboratory equipment. These will be discussed in those parts of the book in which special preparations are described. The student will also need an ordinary "dissecting set," but it is to be presumed that he will have this from previous courses in the biological sciences which he may have taken. If it is the intention of the instructor that the class prepare hand sections, it will be necessary to have section-cutting razors, either available for issue or made part of the students' set of instruments.

2

Fixation and Fixatives

There are a few cases, some of which are described in Chapter 4, where a living animal may be mounted directly on a microscope slide by placing it in a drop of a "mountant" and putting a coverslip on top. This, however, is the exception. It is usually necessary that living forms, or parts of them, be "fixed" in such a manner as to preserve them in the shape which they had during life and "hardened" in order to render them of a consistency suitable to subsequent manipulations. Fixing and hardening agents are usually combined into one solution known as a "fixative," and, from the practical point of view, the worker may require one or more of three functions from the solution employed. These functions are:

1. That the material shall be preserved in the shape it had before fixation.
2. That the nuclear elements of the material shall be preserved.
3. That the cytological elements of the material shall be preserved.

All of these functions can rarely, if ever, be secured from one solution, and each will be discussed separately.

Preservation of External Form. The loss of external form on the part of fixed material is brought about either by the contraction of the animal or by unequal diffusion leading to the distortion of cavities.

The contraction of the animal in many cases may be prevented by preliminary narcotization, which is often essential in the case of invertebrates. Moreover, in such animals as the Rotifera and Bryozoa, the fluid employed must contain an "immobilizing agent" if the external form is to be preserved successfully.

There appear today to be only three immobilizing agents of general value —a temperature between 60° C. and 75° C., osmic acid, and, to a far less extent, mixtures of acetic acid with chromic acid (chromic oxide) and picric acid (trinitrophenol).

The first of these agents—heat—obviously may be added to any known fixative. In the majority of cases, both cytological and histological detail is ruined by its use, yet it remains of great value for many of the marine Hydrozoa which are subsequently intended to serve as wholemounts or museum preparations. Osmic acid is unquestionably the most useful immobilizing agent which has yet been discovered in that cytological, but not always nuclear, detail is well preserved by its use, while many of the lower invertebrates retain far more of their original transparency with this than with any other fixative. This reagent, however, is both expensive to buy and dangerous to use, so that it cannot be recommended to an elementary class.

It is vertebrate embryological material which suffers most from distortion, and the preservation of its external form is best accomplished by solutions containing formaldehyde, potassium dichromate, or potassium dichromate plus sodium sulfate. It must be made quite clear that "distortion" is used here to indicate a change which produces a definite change of "shape." Uniform "shrinking" and "swelling" without an accompanying change of "shape" are perfectly distinct processes; potassium dichromate *(inter alia)* produces the former and formaldehyde the latter, so that combinations of these two or of formaldehyde with Müller's fixing fluid are indicated for the preservation of the external form of delicate mammalian tissues.

Preservation of Nuclear Detail. Much which must be omitted here has been written upon the chemical aspect of this problem. From the practical point of view, the question is bound up with that of penetration. The most universally employed penetrating agent is acetic acid, the swelling action of which is usually restrained by the addition of picric or chromic acid. It should be pointed out that penetration and distortion are usually produced by the same agents, and under this heading must be included many of the ether-alcohol fixatives. The distorting action of these upon whole animals is to a certain extent restrained by the addition of mercuric chloride (cf. Carnoy and Lebrun's fluid, p. 10). In general, however, it is impossible to obtain the finer details of nuclear fixation in entire animals or organs, the shape of which it is desired to preserve.

Preservation of Cytoplasmic Detail. This is largely a question of the chemical or physical coagulation of relatively large masses of protoplasm. This coagulation is brought about by various reagents: e.g., alcohol; compounds of chromium, copper, osmium, and mercury; picric acid; and formaldehyde. The choice of the agent employed must be governed by the particular detail which it is desired to preserve and by the effect which the chosen agent is likely to exert upon the shape and size of the object. The question of transparency should also be included here. Many objects may be preserved conveniently and permanently in neutral formol, where they will retain much of their original transparency; this transparency is usually destroyed by previous fixation in any fluid other than weak osmic acid solutions.

Dichromate Fixatives. Potassium dichromate was one of the earliest substances employed for fixing. It is, however, a very poor protein coagulant. In its original use, in the solution of Müller, it was used in combination with sodium sulfate which is an excellent protein precipitant. Most of the modern dichromate fixative solutions have been derived from Müller, though in most the addition of mercuric chloride has made the sodium sulfate unnecessary.

One of the best known is:

ZENKER'S FLUID:

Water	100	ml.
Potassium dichromate	2.5	Gm.
Mercuric chloride	5	Gm.
Sodium sulfate	1	Gm.
Glacial acetic acid	5	ml.

This is one of the most widely used of the general purpose histological fixatives. It permits very brilliant afterstaining and is in almost universal use in pathological laboratories. Too much should not be made up at one time, for it is not very stable. If large quantities are to be prepared, it is desirable to omit the acetic acid until immediately before use. Pieces should be fixed for about 24 hours and then washed in running water overnight.

For the fixation of botanical specimens, it is customary to use less mercuric chloride. The following is a well-known mixture:

LAVDOWSKY'S FLUID:

Water	100	ml.
Potassium dichromate	5	Gm.
Mercuric chloride	0.15	Gm.
Glacial acetic acid	2	ml.

The lower concentration of mercuric chloride permits specimens to be left in this for a much longer period without danger of becoming hardened.

Many people prefer to add formaldehyde to these mixtures. The best known of these fluids probably is:

HELLY'S FLUID:

Water	90	ml.
Potassium dichromate	2.5	Gm.
Mercuric chloride	5	Gm.
Sodium sulfate	1	Gm.
40% Formaldehyde	10	ml.

This fluid must be made up immediately before use, and fixation should take place in the dark since the presence of light greatly accelerates the reduction of the dichromate by the formaldehyde. It is also better to wash out the material in the dark in a weak (4 per cent) solution of formaldehyde rather than water. This solution is equally applicable to plant and animal tissues which are intended for subsequent sectioning. Some people prefer, particularly for animal tissues, to have acetic acid as well as formaldehyde in these mixtures. The result is often referred to as "Formol-Zenker," though the best known of these mixtures actually is:

HEIDENHAIN'S FLUID:

Water	90 ml.
Potassium dichromate	1.8 Gm.
Mercuric chloride	4.5 Gm.
Glacial acetic acid	4.5 ml.
40% Formaldehyde	10 ml.

This fixative, like that of Helly, should be prepared immediately before use or at least should be prepared as two solutions, one containing the acetic acid and formaldehyde and the other the remaining ingredients. Fixation should also take place in the dark, and the tissues should be washed out in weak formaldehyde in the dark.

Chromic-acid Fixatives. Chromic acid (actually chromic oxide) is widely used in fixatives, usually with the addition of acetic acid. The best-known zoological fixative of this type probably is:

LO BIANCO'S FLUID:

Water	100 ml.
Chromic acid	1 Gm.
Glacial acetic acid	5 ml.

This fixative is particularly adapted for use with small invertebrates and was developed by Lo Bianco for the fixation of marine forms. The fixative should be freshly prepared before use, and the object left in it for about 30 minutes, in the case of an invertebrate larva, to as long as overnight in the case of medium-sized Polychaetes. After fixation, the object should be washed in running water until no further color comes away. Very much weaker solutions are usually preferred by botanists. The one customarily recommended is:

GATES' FLUID:

Water	100 ml.
Chromic acid	0.7 Gm.
Glacial acetic acid	0.5 ml.

This fluid is excellent for the fixation of plant chromosomes in root tips, etc. Specimens should be left in it overnight and then washed out in running water.

The addition of formaldehyde to "chrome-acetic" mixtures is very common in botanical practice. These solutions are known as "Craf" fixatives, a popular example being:

NAVASHIN'S FLUID:

Water	75 ml.
Chromic acid	0.8 Gm.
Glacial acetic acid	20 ml.
40% Formaldehyde	5 ml.

This must be made up immediately before use, or one may prepare it as two solutions, keeping the formaldehyde separate from the chromic acid.

Mercuric Fixatives. Mercuric chloride is often used as the main protein precipitant in a fixative, as well as in combination with dichromate. It has the very grave disadvantage that steel instruments are destroyed instantly on contact with the solutions, so that one must use glass or plastic in handling specimens. There are, however, a number of excellent formulae. One of the best general-purpose fixatives ever invented is:

GILSON'S FLUID:

Water	88 ml.
95% Alcohol	10 ml.
Mercuric chloride	2 Gm.
Glacial acetic acid	0.4 ml.
Nitric acid	1.8 ml.

This is a magnificent fixative for all general zoological uses. Objects may be left in it for many months without becoming unduly hardened, while small objects will be found to be adequately fixed after only a few hours. The fixative should be washed out very thoroughly in 70 per cent alcohol before subsequent manipulations are undertaken. To the beginning student, seeking a general-purpose fixative, this medium is to be recommended above all others.

It occasionally happens that the microscopist desires to fix something which is covered in a very hard shell and, therefore, requires a fixative in which other desirable qualities have to be sacrificed in favor of extremely rapid penetration. One of the best of these mixtures is:

CARNOY AND LEBRUN'S FLUID:

Absolute alcohol	33 ml.
Glacial acetic acid	33 ml.
Chloroform	33 ml.
Mercuric chloride	*to sat.* (about 25 Gm.)

This fluid may be used equally well with a hard-shelled arthropod or with a hard-shelled seed and will penetrate rapidly enough to preserve the whole. It cannot be used satisfactorily with any object containing fat, which will be dissolved, and is usually employed only when the principal interest of the worker is in nuclear fixation.

Picric-acid Fixatives. Picric acid (actually trinitrophenol) has been widely used in the last quarter century as a component of fixatives. The best known formula undoubtedly is:

BOUIN'S FLUID:

Saturated aqueous solution of picric acid	75 ml.
40% Formaldehyde	25 ml.
Glacial acetic acid	5 ml.

It is a great pity that the use of this fixative should have become so widespread, for its only advantage is that objects may be left in it for a long time without becoming unduly hard. It has the disadvantage that picric acid forms water-soluble compounds with many substances found in a cell, so that sections cut from materials fixed in Bouin's fluid frequently show large vacuoles. It is also very difficult to wash the fluid from the tissues; even small traces of picric acid interfere with staining. Gilson's fluid (see p. 10) has all the advantages, and none of the disadvantages, of Bouin's.

Bouin's fluid was recommended by its inventor for the fixation of meiotic figures but has been replaced largely for this purpose by:

ALLEN'S FLUID:

Water	75 ml.
40% Formaldehyde	15 ml.
Glacial acetic acid	10 ml.
Picric acid	1 Gm.
Chromic acid	1 Gm.
Urea	1 Gm.

Small pieces of tissues should be fixed overnight and then washed in 70 per cent alcohol until no more color comes away.

Other Fixative Mixtures. A great variety of other materials has been recommended as fixatives from time to time, but only two are of sufficient interest to be worth repeating here. The first of these is:

KOLMER'S FLUID:

Water	87	ml.
Potassium dichromate	1.8	Gm.
Uranyl acetate	0.75	Gm.
40% Formaldehyde	3.6	ml.
Glacial acetic acid	9	ml.
Trichloroacetic acid	4.8	ml.

This very interesting fixative was developed originally for the fixation of whole eyes but it may be used very profitably in any place in which nerve structures are to be examined subsequently. The salts of uranium are widely used in fixatives intended solely for the central nervous system, but this particular formula is also useful for general purposes. Fixation should take place overnight, and then the material should be washed in running water.

Another very little known but admirable fixative is:

PETRUNKEWITSCH'S FLUID:

Solution A

Water	100 ml.
Nitric acid	12 ml.
Cupric nitrate	8 Gm.

LABORATORY LIBRARY MASS.

Solution B

80% Alcohol	100 ml.
Phenol	4 Gm.
Ethyl ether	6 ml.

Note: One part of A is mixed with three parts of B immediately before use.

This is very nearly as good a general-purpose fixative as the mixture of Gilson and has the advantage over Gilson's that objects fixed in it can be handled with steel instruments before being washed. It is not desirable to leave specimens in it too long (certainly not more than two or three days) and the fixative should be washed out in 70 per cent alcohol.

Removal of Fixatives. Under each of the formulae given above, it has been indicated in what fluid the specimen should be washed to remove the fixative. This simple washing, however, is not always sufficient in the case of fixatives containing mercuric chloride or picric acid. Mercuric chloride occasionally gives rise to long needlelike crystals in tissues. These may be prevented by soaking the specimen, after it has been washed in running water overnight, in:

LUGOL'S IODINE:

Mix 1 Gm. of potassium iodide with 0.5 Gm. of iodine. Add 2 or 3 ml. of water and shake until dissolved. Then dilute to 50 ml. If diluted to 150 ml., this becomes "Gram's iodine."

Note: Iodine is very soluble in strong solutions of potassium iodide and very insoluble in weak solutions.

After the pieces have been soaked in this solution overnight, they should be transferred to either 70 per cent or 90 per cent alcohol in which they should remain until no further color comes away. Specimens so treated never show the fine needlelike crystals after mercuric fixation, but there is no reasonable explanation of why this should be so.

Tissues fixed in picric acid naturally are a bright yellow. All this bright yellow color cannot be removed since some of it is due to the formation of complexes with the proteins. However, there are two methods by which much more of the yellow color can be removed than by washing in alcohol alone. The first is to add a few grains of lithium carbonate to the 70 per cent alcohol in which the specimen is being washed. The lithium salt appears to free some of the bound picric acid. Another method, which is more troublesome but much more satisfactory, is to transfer the specimen to:

LENOIR'S FLUID:

Water	70 ml.
95% Alcohol	30 ml.
Ammonium acetate	10 Gm.

This liberates almost all the bound picric acid and is much the best aftertreatment for picric-acid-stained materials which has appeared in the literature. It might be explained that the objection to the retention of bound picric acid is that it interferes seriously with some forms of staining.

Treating Hard Materials. It so happens that some materials, even after fixation, are so hard that they cannot possibly be cut into sections. This hardening is due either to the presence of calcium in the form of bone or calcareous plates or to the presence of chitin. The removal of bony or calcareous material is not as easy as it sounds, for if one were merely to hang the material in an acid mixture there would be a great hydrolysis of the protein. Therefore, it is necessary to have in the solution, besides acid, something which will prevent hydrolysis and swelling. The most commonly used reagent for this purpose is phloroglucinol. The following is a typical formula:

HAUG'S SOLUTION:

96% Alcohol	70 ml.
Water	30 ml.
Phloroglucinol	1 Gm.
Nitric acid	5 ml.

Note: The phloroglucinol is dissolved in the warm acid, cooled, added to the water, and the alcohol then mixed in. The acid should be heated very gently under a stream of warm water: heating over a flame is likely to cause an explosion.

There is not much use in transferring objects to a vial containing this fluid, for they will fall to the bottom and rapidly exhaust the acid around them. It is much better to hang them with a fine thread of silk from the top of the vial. The decalcification is complete if the object no longer feels hard when pricked with a pin. If there is no part of the object where one may without damage apply the pin, it is very easy to find out if decalcification is complete by having a dentist or physician observe the specimen on a fluorescent screen by x-ray. As soon as decalcification is complete the object should be washed in large volumes of 70 per cent alcohol. Some people claim that phloroglucinol interferes with afterstaining and prefer to restrain the swelling of the tissues with mercuric chloride. The best mixture of this type is:

MCNAMARA, MURPHY, AND GORE'S SOLUTION:

Water	80 ml.
96% Alcohol	10 ml.
40% Formaldehyde	8 ml.
Mercuric chloride	2 Gm.
Trichloroacetic acid	6 Gm.
Nitric acid	1 ml.

This is used in exactly the same way as the solution of Haug, but one must, of course, be careful not to handle the object with steel implements because the mercuric chloride will destroy them.

No really satisfactory method for softening chitinous materials has yet been discovered, though the following is much better than anything else:

JURRAY'S MIXTURE:

Chloral hydrate	50 Gm.
Phenol	50 Gm.

Insects or other chitinous forms are fixed in the fluid of Carnoy and Lebrun and are transferred without washing to Jurray's mixture, where they remain from 12 to 24 hours. Then this mixture is washed out in chloroform, and the objects are embedded in paraffin.

Narcotization. There are many small invertebrates which cannot be made satisfactorily into microscope slides after the process of simple fixation. These forms, such as the majority of small hydroids and worms, are contractile, so that it is necessary to narcotize them before fixation if they are to resemble the living form after mounting. The whole subject of narcotization is very difficult, for it requires great skill to add slowly small quantities of the selected narcotic and then to fix the object at the exact moment that it is completely narcotized but before it is dead. Many marine forms, particularly sea anemones, may be narcotized by adding small quantities of a saturated solution of magnesium sulfate to the water in which they are expanded. There are, however, two mixtures which can be recommended for general purposes. The first of these is:

HANLEY'S SOLUTION:

Water	90 ml.
Ethyl cellosolve	10 ml.
Benzamine hydrochloride	0.3 Gm.

This is an excellent narcotic for small and delicate forms, such as Rotifers and Bryozoa. About one drop per 10 ml. should be added and well mixed with the water in which the creatures are living. After about 10 minutes, further quantities may be added and left until narcotization is complete.

A useful narcotic for less delicate forms is:

GRAY'S MIXTURE:

Grind 48 Gm. of menthol in a mortar with 52 Gm. of chloral hydrate.

This mutual solution of menthol and chloral hydrate is lighter than water. A few drops placed on the surface of the water containing the specimen dissolves slowly, allowing both constituents to act rapidly and safely. This reagent is recommended for Coelenterates of all kinds.

3

Stains and Staining

The majority of objects made into microscope slides today are "stained"; that is, they are soaked in a solution of a dye or dyes, either, in the case of wholemounts, to increase their visibility when mounted in transparent media or, in the case of sections, to provide a color differentiation between the various tissues involved. The staining solutions employed may be divided very broadly into "nuclear stains," which are differentially absorbed by the nucleus and chromosomes, and "plasma stains," which are used to color tissues other than the nucleus. It may be added that, in general, nuclear stains will stain bacteria and are also used in botanical technique for staining xylem.

There are three general methods of staining employed. The first of these is "direct staining" in which soaking the object or section in the stain is sufficient to color the desired structure. This is rarely employed and can be used only with those stains which are entirely specific for the structure which is to be emphasized. It is far more usual to employ a process of "indirect staining" in which the whole object or section is first uniformly stained in the dye selected and then "differentiated" in some solution which removes the dye from all those structures other than the ones which are to be emphasized. The third method of staining is "mordant staining" (from the Latin *mordere,* meaning "to bite") in which some preliminary solution is used which causes the stain to "bite into" a structure without being absorbed by others. These three great categories are by no means sharply divided, for many of the so-called direct stains actually incorporate mordants, and many of the indirect staining solutions may be used by the direct method if they are first greatly diluted with water.

The most commonly employed nuclear stains are hematoxylin, carmine, and a few synthetic aniline dyes. Hematoxylin is a natural coloring matter extracted from logwood and depends for its staining action upon its oxidation products, such as hematein, rather than upon itself. This dye, therefore, must be "ripened" (actually oxidized) either by being left exposed to the air for some time or by having added to it some oxidizing material. Carmine is a complex lake prepared from a dye extracted from the cochineal insect.

Mordant Hematoxylin Staining

The mordant hematoxylin stains are entirely confined to staining nuclei or, more usually, chromosomes. In general, three solutions are required: (1) A mordant, (2) the hematoxylin stain, and (3) a differentiating solution. The two best techniques are those of Dobell and Régaud, though several dozen variants of these have been proposed from time to time.

DOBELL'S MORDANT HEMATOXYLIN STAIN:

Mordanting solution
1% Iron alum in 70% alcohol

Staining solution
1% Hematein in 70% alcohol

Differentiating solution
0.1% Hydrochloric acid in 70% alcohol

All these solutions are stable, and it will be noticed that the staining solution uses hematein, which is an oxidation product, instead of hematoxylin, so that no ripening is required. This method can be used only on very thin sections or on protozoan smears because it gives such an intense coloration that thick sections cannot be differentiated in practice.

PROCEDURE IS AS FOLLOWS:

1. Accumulate thin sections or protozoan smears in 70 per cent alcohol.
2. Transfer them to the mordanting solution for about 10 minutes.
3. Without rinsing, transfer the sections directly to the stain for 10 minutes.
4. Rinse them quickly in 70 per cent alcohol.
5. Differentiate sections in the last solution until only the chromosomes or nuclei remain stained. Differentiation is quite rapid. Remove sections from the differentiating solution at intervals, wash thoroughly in 70 per cent alcohol, and then examine under the microscope. If a number of slides are being stained at the same time, it is desirable to differentiate one carefully, noting the time needed for the operation, and to use this same amount of time to bring the remaining sections through in one batch.
6. Wash them thoroughly in 70 per cent alcohol.
7. Counterstain the sections, if desired, by one of the methods to be described later or mount them in balsam after dehydration and clearing.

REGAUD'S MORDANT HEMATOXYLIN STAIN:

Mordanting solution
5% Iron alum

Staining solution

Water	80	ml.
Hematoxylin	1	Gm.
Glycerin	10	ml.
Alcohol	10	ml.

Differentiating solution

Alcohol	65	ml.
Water	35	ml.
Picric acid	0.5	Gm.

This solution is the best of the general purpose "iron hematoxylins" and should be widely used to stain the nuclei before complex afterstaining. The staining solution contains hematoxylin, and, therefore, must be ripened for some time before use. The most convenient method of doing this is to prepare a 10 per cent solution of hematoxylin in alcohol and then to dilute this with the glycerin in water immediately before use. The alcohol solution of hematoxylin should be at least a month old. This stain is very slow if it is used cold, so that it is customary to heat both the mordanting and staining solutions to about 50° C. before use.

SECTIONS ARE STAINED AS FOLLOWS:

1. Accumulate the sections in distilled water.
2. Transfer the slides to the mordanting solution for 30 minutes at 50° C. or overnight at room temperature.
3. Rinse each slide in distilled water to avoid carrying over too much of the mordant into the staining solution.
4. Transfer the slides to the staining solution for 30 minutes at 50° C. or overnight at room temperature.
5. Transfer the slides to distilled water and wash until no more stain comes away.
6. Dip each slide up and down in the differentiating solution until it appears to be partly differentiated and transfer to tap water until no further color comes away. Then examine the slide under the microscope. If further differentiation is required, repeat the process.
7. Transfer all the slides to tap water until they have turned blue. If the tap water becomes yellow from traces of picric acid, it should be changed or differentiation will continue. Hematoxylin is very sensitive to acids and, as it comes from the differentiating solution, has a reddish brown color, as distinct from the clear blue color required on the finished slide. In many parts of the world, natural tap water is sufficiently alkaline to produce this blue color. If it is not, a pinch of sodium bicarbonate should be added to the coplin jar containing the tap water.

Direct Nuclear Hematoxylin Staining

These stains are not, in general, very satisfactory, for they give neither as sharp staining as the mordant methods nor as good a diffuse stain as the indirect methods.

The method of Ehrlich, however, is so well known that it is given here:

Ehrlich's Acid Alum Hematoxylin:

Staining solution

Water	30 ml.
96% Alcohol	30 ml.
Glycerin	30 ml.
Glacial acetic acid	3 ml.
Hematoxylin	0.7 Gm.
Ammonium alum	*to excess*

Note: Make up the stain by first dissolving the hematoxylin in the acid and alcohol. Then dissolve 1 Gm. of ammonium alum in the water and add this together with the glycerin. Shake well and add about 10 Gm. of ammonium alum to the bottle. Allow to ripen some months, making sure that there are always a few crystals of ammonium alum in the bottom of the bottle. This stain keeps well for about 10 years and works best after it is a year old. Large laboratories, therefore, should make up half a gallon every two or three years in order to have well-ripened material always in stock.

STAIN IS USED AS FOLLOWS:

1. Accumulate the sections in 90 per cent alcohol.
2. Place each slide for ½ to 2 minutes in the staining solution.
3. Remove each slide and drop 90 per cent alcohol on it either from a drop bottle or a pipette until the excess stain has been removed.
4. Transfer the slides directly to alkaline tap water until blue.

Note: If the sections are taken directly from water to stain or from stain to water, a diffuse stain which is very difficult to differentiate will result.

Indirect Nuclear Hematoxylin Stains

These stains are better employed for wholemounts than for sections. They always give a more or less diffuse result, which is excellent for showing up various organs in a wholemount of a small invertebrate or of an embryo. They are excellent, however, for staining botanical sections in which the xylem becomes sharply and clearly stained a bright blue.

Carazzi's Alum Hematoxylin ("Hemalum"):

Staining solution

Water	80 ml.
Potassium alum	5 Gm.
Hematoxylin	0.1 Gm.
Potassium iodate	0.02 Gm.
Glycerin	20 ml.

Differentiating solution

0.1% hydrochloric acid in 70% alcohol

Note: First the hematoxylin should be dissolved in the solution of potassium alum, and then the potassium iodate added; this oxidizes it instantly, taking the place of the long ripening process of other hematoxylin solutions. The glycerin is added last. The stain may be used either for sections or whole-mounts, and the method differs slightly in each case.

METHOD FOR SECTIONS:

1. Accumulate the sections in water.
2. Transfer them to undiluted stain until the nuclei (or, in plant tissues, xylem) is sufficiently stained. This takes 5 to 10 minutes for a plant section but may take several hours for animal tissues.
3. Then wash each slide in distilled water until no more color comes away.
4. Differentiate the sections in the differentiating solution until the nuclei are darkly stained, while the plasma is only lightly stained. In plant tissues it is possible to remove the stain from everything except the xylem.
5. Transfer the sections to alkaline tap water until bright blue.

METHOD FOR WHOLE OBJECTS:

1. Accumulate the objects in water.
2. Transfer the objects to one part of stain plus 10 parts of distilled water for 3 to 10 minutes.
3. Wash them in distilled water until no more color comes away.
4. Differentiate each object in the differentiating solution until the whole object appears a fairly pale pink. Remember, in judging the amount of differentiation required, that the object will appear very much more darkly stained after it has been blued.
5. Transfer the objects to tap water until blue throughout.

Indirect Carmine Nuclear Stains

Both of the carmine formulae given below can be used as direct stains if they are diluted several hundred times with an alum solution. It is not safe to dilute them with distilled water, for there is a risk of the carmine precipitating out, particularly within the cavities of whole animals from which it is almost impossible to remove it subsequently. The best known of all the carmine nuclear stains is:

GRENACHER'S ALCOHOLIC BORAX CARMINE:

Staining solution

Boil together 10 Gm. of borax and 8 Gm. of carmine in 250 ml. of water for about 30 minutes. Cool overnight and filter. Evaporate the filtrate to dryness and then store this powder, labeled as "Borax Carmine Powder." This powder may be dissolved in any strength alcohol from 30 per cent to 70 per cent; in all cases a saturated solution should be employed. This is the original method of making up the solution and is undoubtedly the best.

For those, however, who do not wish to take the time for the latter preparation, a working solution may be made directly in the following manner:

> Boil 2 Gm. borax and 1.5 Gm. of carmine with 50 ml. of water for 30 minutes. Cool and then add 50 ml. of 70 per cent alcohol. This solution should be filtered after two or three days.

METHOD OF USING THE SOLUTION PREPARED FROM THE DRY STOCK POWDER IS AS FOLLOWS:

1. Accumulate the objects in whatever percentage of alcohol is convenient.
2. Make up a saturated solution of the dry powder in alcohol from 10 to 20 per cent stronger than that in which the objects are accumulated.
3. Transfer the objects to stain until they have become a deep red color. This will take from five minutes for an individual protozoan to overnight for a medium-sized flatworm.
4. Transfer the objects to 0.1 per cent hydrochloric acid in alcohol of the same strength as that used for making up the stain. Let them remain in this solution until they become pink and translucent.
5. Dehydrate and mount the objects in the usual manner. This stain is not only good, used in the manner described, for staining wholemounts but is also much the best method of staining the nuclei in blocks of tissue before sectioning if this technique is to be employed.

Grenacher's alcoholic borax carmine is not very satisfactory when diluted and used by the indirect method. For this purpose it is recommended that the following be employed:

MAYER'S CARMALUM:

Staining solution

> Boil together, for 1 hour, 2 Gm. of carmine and 5 Gm. of potassium alum in 100 ml. of water. Cool and filter.

This solution may be used exactly as Grenacher's alcoholic borax carmine for wholemounts or blocks of tissue but it is less satisfactory for this purpose. It is best used after great dilution for direct staining of relatively large invertebrates.

METHOD TO BE EMPLOYED IS AS FOLLOWS:

1. Accumulate the objects in distilled water.
2. Dilute the stain with 5 per cent potassium alum to the required concentration. The larger the object to be stained, the lower the concentration of the staining solution should be. For a relatively small and thin object, such as the prothallium of a fern, a dilution of about 10 to 1 is satisfactory. For a large object, such as a liver fluke, the stain should be diluted until it is only faintly pink.

3. Transfer the object to the stain and leave it until examination shows the nuclei or internal structures to have been stained a fairly dark red, while the other portions are stained only pink. This will take about six hours with a fern prothallium in the dilution described or about three weeks for a liver fluke at the low concentration.
4. Wash the object in running water until all alum has been removed and then mount in the ordinary manner.

Synthetic Nuclear Stains

Of the four stains given below, only celestin blue B and safranin are usually considered to be true nuclear stains, the other stains having been developed for staining bacteria. However, they are all considered together since the bacterial stains often can be used to demonstrate nuclei in material in which conventional stains break down. For example, when one is staining a section of a frog larva or egg, which is heavily loaded with yolk, one has great difficulty in endeavoring to use hematoxylin for the reason that some of the albuminous yolk particles pick up this stain. Either carbolmagenta or crystal violet, however, will demonstrate the nuclei clearly without being picked up by the yolk.

The stain given below is an excellent blue nuclear stain which the author much prefers to hematoxylin because it is impossible to overstain in it.

Celestin Blue B:

Staining solution

Boil together for 5 minutes 0.5 Gm. celestin blue B and 2.5 Gm. iron alum in 100 ml. of water. Cool, filter, and then add to this mixture 14 ml. of glycerin and 2 ml. of strong sulfuric acid.

STAIN IS USED ONLY ON SECTIONS IN THE FOLLOWING MANNER:

1. Accumulate the sections in water.
2. Transfer them to the staining solution until the nuclei are sufficiently stained. This takes anywhere from five minutes to an hour or two, according to the fixative used and the manner in which the sections have been handled previously.
3. Wash sections in water for a few minutes.

It will be seen that this is one of the simplest of all the nuclear staining methods, but it differs from most in that it cannot be used to stain the xylem in plant tissues.

Safranin nuclear staining in English speaking countries is usually confined to botanical specimens, though its use in European countries is widespread for histological purposes. Safranin is not easy to use and is a very slow stain. Undoubtedly the best method is:

Johansen's Safranin:

Staining solution

Methyl cellosolve	50	ml.
95% Alcohol	25	ml.
Water	25	ml.
Sodium acetate	1	Gm.
40% Formaldehyde	2	ml.
Safranin	0.1	Gm.

Note: Dissolve the dye in the methyl cellosolve. Then add the alcohol. Dissolve the sodium acetate in the formaldehyde and water and add these to the dye solution.

Differentiating solution

Identical with that used with Régaud's hematoxylin (see page 16).

STAIN IS USED ON ANIMAL TISSUES AS FOLLOWS:

1. Accumulate the sections in water.
2. Transfer the sections to stain and let them remain there from 24 to 48 hours (or even longer) until the nuclei are darkly stained.
3. Dip the slide up and down in the differentiating solution until the unwanted stain has been removed from the cytoplasm.
4. Wash it in running water to remove the picric acid.
5. Mount the section in the usual manner.

The stain given below is the basis for about 90 per cent of all bacteriological techniques:

Ziehl's Carbolmagenta:

Staining solution

Grind together 1 Gm. of magenta ("basic fuchsin") with 10 ml. of 90 per cent alcohol and 10 Gm. of phenol. When this mixture has been reduced to a paste, take 100 ml. of water and rinse out the mortar, using 10 successive 10-ml. batches. These batches should be accumulated, left a few hours, and then filtered.

The use of this stain for bacteria is described on page 104. If it is to be used for staining nuclei in either animal or plant sections, it is necessary only to transfer the section from water to the stain for 10 to 20 minutes and then to differentiate as long as is required with 1 per cent acetic acid.

It is sometimes necessary to stain bacteria blue, either as a contrast to the red color produced by Ziehl's solution or in order to differentiate bacteria which have been decolorized. Much the best solution for this purpose is:

Lillie's Ammonium Oxalate Crystal Violet:

Note: This solution is often called "Hucker's ammonium oxalate crystal violet or Hucker-Conn crystal violet."

Staining solution

Dissolve 2 Gm. of crystal violet in 20 ml. of 95 per cent alcohol. Dissolve separately 0.8 Gm. of ammonium oxalate in 80 ml. of distilled water. When both solutions are complete, add the oxalate solution to the dye solution.

The proportions used here are those given by Lillie.

The use of this solution in staining bacterial films is described on page 100 and its use for the staining of nuclei is identical with the method outlined for Ziehl above.

Plasma or Contrast Stains

Wholemounts are almost invariably stained in one color only since sufficient nuclear stain usually remains dispersed throughout the plasma to provide adequate visibility. The cytoplasm of sections, however, is usually stained a contrasting color, both to render the nuclei more apparent and to emphasize the general structure. Sometimes two or more colors may be employed. Single contrasts are usually perfectly adequate, and the following may be recommended:

EOSIN Y:

Make up as a 0.5 per cent solution in distilled water.

ETHYL EOSIN:

Make up as a 0.5 per cent solution in 95 per cent alcohol.

Both these eosins give very much the same shade and are the conventional contrast to hematoxylin- or celestin blue B-stained sections. The choice between water and alcohol is a matter of individual preference.

Another popular contrast stain is:

FAST GREEN FCF:

Make up as a 0.1 per cent solution in 90 per cent alcohol.

This is sometimes used as a contrast for hematoxylin, for which purpose it is not as satisfactory as the eosins, but it is very widely employed as a contrast stain to red nuclei stained either with safranin or carmine.

Double Contrast Stains

It is no more trouble to use a single solution which will stain different tissues various shades than it is to use a simple solution. Two solutions can be confidently recommended. The first of these is:

VAN GIESON'S STAIN:

Staining solution

To 100 ml. of a saturated solution of picric acid in water, add 0.05 Gm. of acid fuchsin.

STAIN IS USED AS FOLLOWS:

1. Collect sections, with the nuclei stained blue, in tap water.
2. Stain them from 2 to 5 minutes in the staining solution.

3. Give each slide an individual quick rinse in tap water and pass it directly to 96 per cent alcohol.
4. Keep the slides in motion in 96 per cent alcohol until they are dehydrated.
5. Then rinse each section once or twice in absolute alcohol and transfer it to xylene.
6. As soon as the section is clear, mount it in balsam.

This stain relies for its effect on the fact that muscular tissues retain the yellow color of the picric acid more readily than the other connective tissues. It is the best known of the double contrast solutions but has certain serious disadvantages. In the first place, picric acid tends to remove hematoxylin from the nuclei, so that one must overstain considerably in the hematoxylin if one is to secure adequately blue nuclei in the finished mount. The second major objection is that picric acid is soluble to a certain extent in xylene so that the yellow color is gradually extracted. Sections stained by this method and mounted in balsam are rarely of very much use a year after they have been prepared. To avoid these objections, the author much prefers:

GRAY'S DOUBLE CONTRAST STAIN:

Staining solution

Water	100 ml.
Orange II	0.6 Gm.
Ponceau 2R	0.4 Gm.

SOLUTION IS USED AS FOLLOWS:

1. Accumulate sections, with nuclei stained either in hematoxylin or in celestin blue B, in tap water.
2. Transfer the slides to the staining solution for 2 minutes.
3. Remove each slide individually from the stain, drain, blot, and then dip up and down in absolute alcohol until sufficiently differentiated. The completion of differentiation and the completion of dehydration usually coincide.
4. Then transfer each slide to xylene and mount in the ordinary manner.

This stain gives a good range of red-orange and gold shades on most histological sections and is no more difficult to use than a simple solution of eosin.

Double contrast stains for sections in which the nuclei have been stained red are not very common, though the following, originally designed for use with heavily yolked embryonic material, is really excellent.

SMITH'S PICRO-SPIRIT BLUE:

Staining solution

Absolute alcohol	100 ml.
Picric acid	1 Gm.
Spirit blue	*Enough to saturate*

STAIN IS USED AS FOLLOWS:

1. Take sections of material which have been bulk stained in carmine through the ordinary procedures as far as absolute alcohol.
2. Transfer them to the staining solution for 2 minutes and rinse individually in absolute alcohol until sufficiently differentiated.
3. Then transfer the slides to xylene which stops further differentiation. This gives good differentiation of a large number of tissues. On embryonic material it is particularly effective, for the nuclei are red, yolk and yolk granules are yellow-green to green, while yolk-free cytoplasm is a clear blue.

Complex Staining Techniques for Animal Tissues

Complex staining solutions are those in which a series of stains, mordants, and differentiating solutions are used one after another in such a manner that the nuclei and all the elements of the plasma are stained in sharply contrasting colors. The stains used in botany, naturally, are quite different from those used in histology and pathology and are given immediately following this section. Many hundred complex stains are in the literature and will be found in Gray's *Microtechnique,* where several hundred more stains of specific application are also given. Here it is intended to present only three stains which are so simple to use and so excellent in their results that they should be known to everyone. The first of these is:

MALLORY'S TRIPLE STAIN:

First staining solution
1% Acid fuchsin
Differentiating and mordanting solution
1% Phosphotungstic acid
Second staining solution

Water	100	ml.
Methyl blue	0.5	Gm.
Orange G	2	Gm.
Oxalic acid	2	Gm.

METHOD OF USE:

1. Accumulate sections for staining in water.
2. Stain them in the first staining solution for 2 minutes.
3. Rinse the slides thoroughly in water.
4. Transfer them to the phosphotungstic acid for 2 minutes.
5. Give the slides a very quick rinse in water. The purpose is to remove the phosphotungstic acid from the slides, not from the sections.
6. Transfer to the second staining solution for 15 minutes.
7. Wash the slides in water until no more color comes away.

8. Take each slide individually and dip it up and down in absolute alcohol until it is differentiated. This may be seen clearly with the naked eye, for the slide, which is a muddy purple when differentiation starts, suddenly clears to show bright blue and bright red areas.
9. Transfer the slide to xylene which stops differentiation.

When using this stain for the first time, it is well to transfer sections to xylene and examine them under the microscope before differentiation is complete and then to put them back into absolute alcohol to complete the differentiation. A successful slide will show nuclei in red, cartilage and white fibrous connective tissue in blue, nerves and glands in various shades of violet, muscle in red, and erythrocytes and keratin in orange. The only disadvantage of the technique is the rapidity with which the blue color is removed in absolute alcohol, so that differentiation must be watched very carefully. The dyes used are extremely sensitive to alkali, so that the slides, if they are to be kept for some time, should be mounted in a very acid medium. A procedure which has been recommended is to keep a saturated solution of salicylic acid in xylene and to dip each coverslip in this before applying it to the finished preparation. This is much less trouble than, and just as effective as, making up a special salicylic acid balsam.

Another complex contrast stain, in which the nuclei first must be stained with hematoxylin, is:

PATAY'S TRIPLE STAIN:
  First staining solution
    1% Ponceau 2R
  Differentiating and mordanting solution
    1% Phosphomolybdic acid
  Second staining solution
    0.5% Light green in 90% alcohol

METHOD OF USE:

1. Stain the sections in Carazzi's hematoxylin (see page 18) and differentiate until not only the nuclei but also the cartilage remains blue. Wash the sections in alkaline tap water until they are blue and then wash thoroughly in distilled water to get rid of all the alkali.
2. Put the sections in the first stain for 2 minutes.
3. Rinse them briefly in water.
4. Place them in the differentiating and mordanting solution for 2 minutes.
5. Rinse sections briefly in water.
6. Transfer them to 95 per cent alcohol until dehydrated.
7. Transfer each section individually to the second staining solution for 30 seconds.

8. Rinse each slide individually in absolute alcohol until no more color comes away.
9. Transfer the slides to balsam and mount in the usual manner.

This is one of the most brilliant of all the triple stains, and a successful preparation shows the nuclei in blue-black and the cartilage in clear blue. Other connective tissues are green, white fibrous connective tissue being light green, and bone is a most brilliant green in contrast to the orange muscle and blue cartilage. Red blood cells are yellow and most nerve tissues are colored a neutral grey. This stain is little more trouble to apply than Mallory's, but the results are both more brilliant and more permanent.

Many complex stains rely on the fact that a mixture of eosin and methylene blue solutions gives rise to a precipitate which, though insoluble in water, is soluble in methyl alcohol. These stains are used mainly for blood films, not only to differentiate the cell types but also to display the parasites present. The best known of these is:

WRIGHT'S STAIN:

The preparation of this stain is very complicated. (It is described in Gray's *Microtechnique.)* It is strongly recommended that the solution be purchased and used as it comes from the bottle.

METHOD, AS APPLIED TO AIR-DRIED BLOOD SMEARS, IS AS FOLLOWS:

1. Flood the stain on the slide from a drop bottle and leave for 2 minutes.
2. From another drop bottle, add distilled water drop by drop until a green scum forms on the surface of the stain.
3. Wash slide in distilled water until no further color comes away.
4. Dry and examine the slide.

This is the standard stain used for counting and differentiating leukocytes. There are hundreds of variations to this method, and also hundreds of other methods of staining blood. Wright's stain is, however, the basic method from which most others have been derived.

Complex Botanical Stains

Most of the combinations used for zoological staining, such as hematoxylin-eosin, can also be used in plant structures, where it is desired to differentiate between the nucleus and the cytoplasm. It is only a matter of convention that the safranin and light green combination, so little used in zoological techniques, is preferred by most botanists for their material.

The complex stains are an altogether different matter, since the chemical nature of plant structures is naturally different from that of animals. The best of these complex botanical stains is:

JOHANSEN'S QUADRUPLE STAIN:

First staining solution

Johansen's safranin (see page 22)

Second staining solution

1% Methyl violet 2B

First differentiating solution

95% Alcohol	30 ml.
Methyl cellosolve	30 ml.
Tertiary-butyl alcohol	30 ml.

Third staining solution

Mix 6 ml. of methyl cellosolve with 6 ml. clove oil and saturate this mixture with fast green FCF. Filter the saturated solution and add to it 35 ml. of 95 per cent alcohol, 35 ml. of tertiary-butyl alcohol, and 12 ml. of 1 per cent acetic acid.

Second differentiating solution

95% Alcohol	50 ml.
Tertiary-butyl alcohol	50 ml.
Glacial acetic acid	0.5 ml.

Fourth staining solution

Prepare separately saturated solutions of orange G in methyl cellosolve and 95 per cent alcohol. Mix fifty ml. of each solution.

Third differentiating solution

Clove oil	30 ml.
Methyl cellosolve	30 ml.
95% Alcohol	30 ml.

Special dehydrating solution

Clove oil	30 ml.
Absolute alcohol	30 ml.
Xylene	30 ml.

STAIN IS USED AS FOLLOWS:

1. Accumulate sections mounted on slides in 70 per cent alcohol and place them in the first staining solution for 1 to 3 days.
2. Wash slides in running tap water until no more color comes away.
3. Transfer them to the second staining solution for 10 to 15 minutes.
4. Rinse slides in running tap water.
5. Differentiate them for 10 to 15 seconds in the first differentiating solution.
6. Stain sections in the third staining solution from 10 to 20 minutes or until sufficient green dye has been absorbed.
7. Rinse them for 5 to 10 seconds in the third differentiating solution.
8. Place sections in the fourth staining solution from 3 to 5 minutes or until the cytoplasm of the cells has become bright orange.
9. Dip them up and down 3 or 4 times in the special dehydrating solution.
10. Transfer slides to xylene and mount in the ordinary manner.

In a successful preparation of this type, chromosomes and lignified cell walls are stained bright red, the contents of the cells having purplish resting nuclei against a bright orange cytoplasm. There is a very vivid, but somewhat variable, differentiation of the remaining structures. Parasitic fungi are particularly well shown in bright green whether they are penetrating cytoplasm or the lignified portion of the cell wall.

Stains for Special Purposes

The number of stains for special purposes is legion. They belong properly in the field of the specialist in the particular tissue which they demonstrate. There are, however, a few which are sufficiently interesting and simple to justify their inclusion in a beginner's handbook.

Fats. Most fats are normally dissolved from tissues in the course of embedding in wax. If, however, frozen sections are made by the technique described on page 71, it is possible to demonstrate differentially the fat globules by soaking the sections in an alcoholic solution of a fat soluble, but water insoluble, dye. The classic method is to use a saturated solution of sudan IV in 70 per cent alcohol. This stains fat globules red. A blue color may be obtained by using a saturated solution of oil blue N in 60 per cent isopropyl alcohol. Sections stained by this method cannot be dehydrated and should be mounted in Farrants' medium (see p. 35).

Skeletons. It is often useful, in the study of embryos or very small vertebrates, to be able to stain the skeleton differentially.

Specimens, such as fish fry, which have bony skeletons should be preserved in 70 per cent alcohol made slightly alkaline by saturating it with borax. When they are thoroughly hardened, a 0.5 per cent solution of alizarin red S in absolute alcohol is added in the proportion of 1 ml. of stain for each 100 ml. of preservative. The alizarin forms a red lake with the calcium in the bones. When the bones are red enough, the surplus stain is washed out of the other tissues with alkaline alcohol. The embryos are then dehydrated and mounted in balsam.

Cartilaginous skeletons cannot be directly stained, as are bones, but may be indirectly stained in toluidine blue. The embryos are fixed in any fixative not containing picric acid—mercuric mixtures are preferred by most people—and thoroughly washed. They are soaked for 24 hours in:

VAN WIJHE'S STAIN:

70% Alcohol	100 ml.
Hydrochloric acid	0.1 ml.
Toluidine blue	0.1 Gm.

Then they are differentiated in 0.1 per cent hydrochloric acid in 70 per cent alcohol until no more color comes away. Dehydration and mounting in balsam will show the cartilage alone stained clear blue.

4

Dehydrating and Clearing

Any object which is to be mounted in a resinous medium or embedded in paraffin for section cutting will have to be "dehydrated" and "cleared" before either of these processes can take place. Neither balsam nor paraffin are miscible with water or even with alcohol. The processes of dehydration and clearing involve soaking the specimen in some material which will extract the water from it, and which itself is miscible with some other material in which balsam and wax can be dissolved. The first of these reagents, used to extract water from the specimen, is known as a dehydrating agent. The second reagent, used for the removal of the dehydrating agent and in which balsam or wax must be soluble, is known as a clearing agent, because most of the chemicals used have a relatively high index of refraction and, thus, cause the object to become more transparent.

The commonest reagent used for dehydrating is ethyl alcohol, which is available in most laboratories both as neutral grain spirits (95 per cent alcohol) and absolute alcohol (100 per cent alcohol). The removal of the last 5 per cent of water from neutral grain spirits is a very expensive operation, so that 95 per cent alcohol should be used wherever possible.

Were the majority of specimens merely to be thrown in 95 per cent alcohol, the violent diffusion currents which would be set up would result in the collapse of cavities or in the distortion of the specimen. It is customary, therefore, to use these alcohols as a graded series. It is conventional today to employ the series of 30 per cent, 50 per cent, 70 per cent, 90 per cent, and 95 per cent and to pass the specimen from one of these strengths to the next, leaving it in each sufficiently long to become impregnated. This series is not reasonable, for there is a much greater and more violent diffusion current when a specimen is passed from water to 30 per cent alcohol than there is when a specimen is passed from 70 per cent to 90 per cent alcohol. The author much prefers to use the series of 15 per cent alcohol, 40 per cent alcohol, 75 per cent alcohol, and 95 per cent alcohol, and would recommend this for the beginning student. This series more nearly represents the intention of the worker, which is to subject the specimen to a graded series of stresses rather than to a graded series of alcohols.

It is doubtful whether or not it is necessary even to use a series of alcohols when the object to be dehydrated is a thin section attached to a slide. The only purpose of using an intermediate concentration of alcohol between water and 95 per cent is to avoid the rapid dilution of the latter by the water carried over on the surface of the slides. This difficulty can be avoided by using two jars of 95 per cent alcohol. It must be understood, of course, that the section will take just as long to dehydrate as when a series is employed, but one avoids the difficulty of transferring the section through many jars.

Within the last decade there have been introduced a number of substitutes for ethyl alcohol, some of them intended for use in circumstances where ethyl alcohol is hard to obtain and others intended to serve the purpose of a "universal solvent," miscible alike with water, balsam, and wax. The best of the alcohol substitutes is undoubtedly cellosolve (ethylene glycol monoethyl ether). This substitute has many advantages over ethyl alcohol for purposes of dehydration but cannot be substituted for it in the preparation of many stains and staining solutions. It is less volatile than alcohol, so that, if left in an uncovered dish, it does not evaporate so rapidly. It is also somewhat less hygroscopic, so that, under the same circumstances, it does not lose its strength. It has the disadvantage that it is more viscous than alcohol and tends to give rise to greater diffusion stresses, therefore, it must be used in a more extended series of graded mixtures if delicate wholemounts are to be passed through it. However, for the handling of tissues intended for embedding in paraffin and, above all, for the routine handling of tissues in pathological laboratories, cellosolve is strongly recommended.

The only one of the "universal solvents" which has found any general acceptance is dioxane (diethylene dioxide). This solvent is readily miscible with water and with balsam, and is slightly less miscible with molten paraffin. Specimens, therefore, can be transferred directly from water to dioxane—a graded series is necessary should they be delicate—and, after having been thoroughly impregnated with dioxane, transferred directly either to the mounting medium or to a bath of molten paraffin for impregnation. In spite of the apparent simplicity of the use of this solvent, there are certain great objections. The first of these is the toxicity of dioxane vapor to humans. Dioxane is a cumulative poison and has been shown to affect seriously the function of both the liver and the kidneys. This does not matter very much where it is used by an individual who knows himself to be free from hepatic or renal disorder, particularly where he is exposed only to low concentrations of the vapor for relatively short periods. However, it militates heavily against the use of this reagent in large classes where the instructor is responsible for the health of individuals without knowing their physical idiosyncrasies. Another disadvantage is in the heavy diffusion stresses which are set up when materials are transferred from dioxane to molten paraffin. This is not so important when dealing with tissue blocks for routine histological examination but it is almost impossible to get a good section of a 72-hour chick embryo, for example, using this shortened dioxane technique.

Clearing agents, which remove the dehydrating agent from the tissues and leave them in condition either for mounting in balsam or embedding in paraffin, are of two main types. For mounting in balsam, it is customary to use one of the essential oils or their synthetic equivalents. The advantage of these materials is that many of them are readily miscible with 90 per cent to 95 per cent alcohol and, therefore, are capable of removing the last traces of water which may be left in the specimen after imperfect dehydration.

In the author's opinion, the best clearing agent for general use before making wholemounts is terpineol ("synthetic oil of lilac"). This material is readily miscible with 90 per cent alcohol and has the additional advantages that it has neither an unpleasant odor nor does it render objects brittle. It is, however, more customary today to recommend clove oil. This has the advantage of being much more fluid than terpineol and the disadvantages of a very pungent odor and the tendency to make small objects brittle. The latter is sometimes an advantage, as when one is endeavoring to remove appendages from small arthropods, but on other occasions it is very annoying. Many other oils have been recommended from time to time, but these two between them will be satisfactory for making a wide variety of wholemounts. Under no circumstances whatever should an essential oil be used to clear objects intended for embedding in paraffin, because the oil is almost impossible to remove completely and will destroy the good cutting qualities of the embedding medium selected.

For clearing or dealcoholizing objects intended for embedding in wax, it is customary to use a hydrocarbon, and xylene is in almost universal employment at the present time. From the point of view of physical properties, there is little choice between benzene, xylene, and toluene; the author considers that the first of these has a distinct advantage in that it tends to render objects less brittle. All three are equally miscible with molten paraffin, but none is as good a solvent of solid paraffin as is chloroform. Both the hydrocarbons and chloroform are very sensitive to water, so that it is essential that an object be completely dehydrated in alcohol or dioxane before being transferred to one of these clearing agents. It occasionally happens that it is impossible to provide perfect dehydration, and then one is forced to utilize the "coupling" properties of phenol, which is usually employed in the following mixture:

CARBOLXYLENE:

Xylene	70 ml.
Phenol	30 Gm.

The exact proportion of the ingredients varies somewhat according to the opinion of the worker, that given above being the most usual. Specimens may be taken directly from 50 per cent alcohol into this reagent which, however, must be removed thoroughly by washing in pure xylene before the specimen is transferred to wax. It should be pointed out to the inexperienced student that phenol is capable of giving a most unpleasant burn and that carbolxylene itself should be kept away from the hands under all circumstances. This reagent is also useful when one is endeavoring to clear objects in hot humid weather in summer. The alcohol used for the dehydration absorbs water so rapidly under these circumstances that it is almost impossible to clear satisfactorily in xylene alone.

In the case of small objects, no special precautions need to be observed in the technique of dehydrating. That is, one may merely place the object at the bottom of the tube, fill the tube with the required strength of alcohol, and change this for stronger alcohol as often as becomes necessary. This technique, however, cannot be employed if one is dealing with large objects, since there is a tendency for the water abstracted from the object to accumulate at the bottom of the tube and thus prevent satisfactory dehydration. Objects of a size larger than a grain of corn should always be suspended from the top of the vessel containing the dehydrating agent either in a small bag of cloth or, where the nature of the object permits, from a hook inserted into the cap of the tube or jar. These remarks do not apply when one is dehydrating with cellosolve, which is denser than water.

The technique of clearing specimens in a hydrocarbon or in chloroform before embedding in wax is exactly the same as dehydrating, and no special precautions need be observed. In the case of delicate objects intended for wholemounting, however, it is necessary to provide some gradient between the alcohol and the essential oil if one is to avoid distortion. The simplest way of doing this is to use a flotation method similar to that used by a bartender when he makes a "pousse-café." That is, one, first of all, pours a layer of the oil on the bottom of the tube and then very carefully, with the utmost precautions against mixing, floats a layer of alcohol on top of this. The object is transferred from the alcohol to the upper layer of alcohol in the tube through which it drops to the junction of the two fluids. As soon, however, as it has become partially impregnated with the clearing agent, it sinks to the bottom of the tube. As it lies there, columns of alcohol will be seen rising from it. When the alcohol has ceased to rise, the object is extracted with a pipette with care so as not to get any alcohol into the pipette, and then transferred to a tube or dish of the pure essential oil where clearing will be completed in a few minutes.

There is no more common cause of failure in the preparation of microscope slides than imperfect dehydration and clearing. It is a sheer waste of time to endeavor to embed an object in paraffin unless all the water and all the alcohol have been removed from it, and it is a waste of time to endeavor to impregnate it with xylene unless all the water has been removed from it by alcohol. It is quite impossible to give any particular time schedule for any particular object; experience is the only guide. It is easy to see, however, when an object has not been dehydrated or cleared perfectly. The least trace of milkiness—as distinct from opalescence—is an indication that the water has been imperfectly removed in alcohol, so that the object cannot be cleared properly. If this slight milkiness is observed, the object must be returned to absolute alcohol until such time as all the water has been removed. There is no simple method of determining when all the alcohol has been removed by xylene. It is usually safer to use three changes, allowing ample time in each, than to embed an object which has been cleared imperfectly and which will be impossible to section subsequently.

Perfect clearing is just as essential in objects intended for wholemounts and is much easier to determine since the essential oils are of sufficiently high refractive index to make a properly cleared object appear glass-clear. No further clearing will take place in Canada balsam, so that, unless the object appears perfect in oil, it is a waste of time to mount it.

A final point to remember is that dehydrating agents must of necessity be hygroscopic, and that they will dehydrate the air as readily as they will dehydrate the specimen. It is desirable, therefore, either to use fresh absolute alcohol from an unopened bottle or, if one is not using the whole bottle at a time, to keep a layer of some good dehydrating agent at the bottom of the bottle. The best dehydrant for use in absolute alcohol is anhydrous copper sulfate, for this not only absorbs water readily but also indicates, by changing from white to blue, when it is becoming exhausted. Anhydrous calcium sulfate, in the form commercially known as Drierite, is a somewhat better dehydrating agent but cannot be used as an indicator in alcohol.

One may also anticipate that both the hydrocarbons and the essential oils used for clearing will be in a water-saturated condition when purchased. These, therefore, should always be dehydrated as soon as they have been purchased—preferably using Drierite—but, since they have little tendency to absorb moisture from the air, it is not necessary to keep them in bottles containing a dehydrating agent.

5

Mounts and Mountants

The final mounting of an object or section for microscopical examination consists of cementing it between a slide and coverslip in a medium or "mountant" which will preserve it permanently and retain it in a sufficiently transparent condition for study. There are two kinds of mountants: (1) Those which are miscible with water and to which objects may be transferred directly and (2) those which are not miscible with water and which require that the object be prepared by dehydration and clearing as described in the last chapter.

All too little attention is paid to the water-soluble mountants which are really much more suitable for mounting many objects than is the balsam usually employed. Stained objects, of course, cannot be mounted in these aqueous media, but a large number of small invertebrates, particularly arthropods, make better preparations in gum media than they do in balsam.

There are two types of gum media: (1) Those of relatively low index of refraction which do not render the objects placed in them very transparent and (2) those of a very high index of refraction for use in circumstances where a transparency almost equivalent to a balsam mount is required. The two most useful of the low index of refraction media are:

FARRANTS' MEDIUM:

Water	40	ml.
Gum acacia	40	Gm.
Glycerin	20	ml.
Phenol	0.1	Gm.

The original formula used a saturated solution of arsenous oxide as a preservative, but phenol is much better. One may use, in point of fact, any preservative to inhibit the growth of fungi. This medium is rather difficult to make up, for it is almost impossible to obtain a sample of gum acacia which is not contaminated with pieces of bark, sand, and dirt. A 50 per cent solution of this material, even when diluted with the glycerin, is very difficult to filter, and it is much better to secure this medium from a supplier of materials than to make it up oneself. A medium which avoids this difficulty by using synthetic materials is:

GRAY AND WESS' MEDIUM:

Polyvinyl alcohol	2 Gm.
70% Acetone	7 ml.
Glycerin	5 ml.
Lactic acid	5 ml.
Water	10 ml.

First make a smooth paste of the dry alcohol with the acetone. Then mix half (5 ml.) of the water with the glycerin and lactic acid and stir this into the paste. Add the remaining 5 ml. of water drop by drop, stirring constantly. The mixture thus produced is cloudy at first but will become transparent if heated on a water bath for about 10 minutes.

The advantage of this medium is that it sets fairly rapidly to a tough consistency, so that one may handle a slide made with it within half an hour. A slide made in Farrants' medium may require drying for several days before it is safe to stand it on edge.

The best known of the high, refractive-index, watery media is:

Berlese's Medium:

Water	10 ml.
Glacial acetic acid	3 ml.
Dextrose syrup	5 ml.
Gum acacia	8 Gm.
Chloral hydrate	75 Gm.

Mix the water with the acid and the dextrose syrup. Dissolve the gum acacia in this mixture. This will take a week or so, and the material should be stirred at intervals with care so as not to include too many air bubbles. When solution of the gum acacia is complete, the chloral hydrate is added and stirred to solution.

This mixture suffers from the same disadvantage as does Farrants' in that it is difficult to secure clear gum acacia. There is, however, no other medium of such high refractive index which is so suitable for mounting small arthropods. The chloral hydrate seems to act as a narcotic, so that a small specimen placed in this medium usually expands into a relaxed condition with all the appendages well displayed for examination. If difficulty is encountered in drying the medium, it is probably due to the very large quantity of chloral hydrate. In those laboratories where the atmosphere is commonly humid, it would be well to reduce the quantity of chloral hydrate to 60 Gm.

There are two main types of resinous mounting media: (1) Those into which the object may be placed directly from alcohol and (2) those for which the object first must be cleared. The former type of medium is commonly referred to as a "neutral" mountant and is used almost exclusively for mounting stained blood films which are very sensitive to the acid which inevitably develops in balsam mounts. The best known of these neutral mountants is a proprietary compound of secret composition known as euparal. This may be obtained commercially, but for those who prefer to prepare their own solutions a very excellent substitute is:

MARINE BIOLOGICAL LABORATORY
LIBRARY
WOODS HOLE, MASS.

Mohr and Wehrle's Medium:

Camsal	10 ml.
Gum sandarac	40 Gm.
Eucalyptol	20 ml.
Dioxane	20 ml.
Paraldehyde	10 ml.

Camsal is a viscous fluid produced by the mutual solution of equal quantities of camphor and phenyl salicylate (salol). It is well to make up more of this than is required for the preparation of the medium, since it may be used for clearing whole objects which are intended for mounting in a medium of this type. There is a superstition that a variety of euparal, known as green euparal, preserves the color of hematoxylin stains better than does the plain material. The formula given above may be turned into an imitation of green euparal by adding as much of a solution of copper oleate in eucalyptol as needed to produce the required color.

The most usual resinous mounting medium is Canada balsam. This is the natural exudate of the balsam tree *(Abies balsamea)*. Unfortunately, commercial samples are often contaminated with the exudates of other resinous trees, which render the material less suitable for the preparation of microscope mounts. This balsam, as all other balsams, is a solution of a "resin" in turpentine and contains, in addition, a series of higher-boiling-point hydrocarbons, which serve as "plasticizers" to render it less brittle when it dries. The material is commercially obtainable in two forms. "Natural balsam" is the thick sticky material as it comes from the tree and should always be used in the preparation of wholemounts. The other form is "dried balsam" which has been heated to the extent that the turpentines are driven off, but the natural plasticizers remain. This material is dissolved in xylene to a suitable consistency and is used for mounting sections. The dried balsam, unfortunately, is often carelessly prepared by suppliers who heat it to the point where the natural plasticizers, as well as the turpentines, are driven off. This results in a very brittle compound, and slides made with it are quite likely to flake off the coverslip a year or two after they have been mounted. Therefore, specimens purchased should be examined carefully by pressing them with the thumb nail. If, on pressure with the thumb nail, one of the pieces of dried balsam cracks into a powdery material, the sample should be rejected. The desirable consistency is that in which the thumb nail will just manage to mark a piece without any shattering. It is usually worth while to prepare one's own dried balsam from the natural balsam by tipping a pound or two into a shallow metal container (a baking dish is excellent) and placing this on a hot plate, taking due precautions against fire. At intervals a small drop should be removed on the end of a glass rod and placed on an ice-cold sheet of metal where it will harden. The process of evaporation should be stopped when the test specimen shows the desired characteristics. This dried balsam is usually dissolved in the proportion of 60 Gm. of balsam to 40 Gm. of xylene, although some people, including the author, prefer to substitute chloroform for xylene.

A good many attempts have been made to produce a synthetic resin which will have the desirable qualities of balsam without having the disadvantage of becoming yellow or turning acid with age. At the present time none of these is altogether satisfactory. There is also the grave risk, in specifying a formulation of this nature, that the proprietary resins mentioned will no longer be on the market when one endeavors to prepare the recommended mountant. At least to the beginner it is strongly recommended that he confine himself to natural balsam for wholemounts and a solution of dried balsam in a volatile material for mounting sections.

6

Making Wholemounts

The last few chapters have surveyed a few of the reagents and processes used in the preparation of microscope slides. It is now time to turn to the preparation of the slides themselves. Those described in this book are prepared in any one of three ways. First there are "wholemounts" which, as the name indicates, are mounts either of whole organisms or of parts of organisms which are sufficiently small or transparent to be studied without specialized treatment. Slides of the second type, described in the next chapter, are "smears" in which a thin layer of some fluid intended for study is spread upon a slide and there stained and mounted. Preparations of the third type are "sections" which are thin slices cut from an object either too thick or too complex to be studied as a wholemount; the preparation of sections is described in Chapter 8. Wholemounts are the easiest of all slides to prepare and should be the first to be made by the beginner.

Temporary Wholemounts. Temporary wholemounts are prepared by every student for the purpose of examining material under a microscope. No object should ever be examined on a slide until the coverslip has been placed over it. The purpose of the coverslip is not only to prevent water from condensing on the lenses but also to provide a flat surface for observation. Temporary wholemounts are usually prepared in water by the simple process of taking a drop of the fluid containing the material to be examined, such as living Paramecium, and lowering the coverslip on the surface. This is perfectly satisfactory, providing it is not necessary to observe the objects for long periods. After a time, however, the water evaporates from the edges of the coverslip, which crushes the material. This may be avoided readily by slowing the evaporation with petroleum jelly, a little ring or square of which is built up in the center of the slide before the drop is placed in position. The coverslip is pressed down very gently, until it is seen to be sticking to the petroleum jelly.

There are many tricks which may be used to examine animals that move too rapidly for study. One of the simplest of these is to place a piece of lens paper on the slide and then to place a drop of the culture under examination on this. The addition of a coverslip causes the fibers of the lens paper to make, as it were, a series of little compartments in which the animals become trapped. Another useful device is to mix thoroughly a culture of the animal in question with an equal volume of 0.5 per cent agar. This thickens the liquid sufficiently to slow down most forms.

The term wholemount, however, usually means a permanent preparation made in some medium which will preserve the object and hold the coverslip in place. These mounting media are either water miscible, in which case most objects can be placed in them directly, or resinous, requiring extensive preparation of the object. Each type will be described in its turn.

Mounting in Gum Media. The simplest preparations are those made in water-miscible mountants, which are of far wider utility than is usually realized because of a complete mental block on the part of most microscopists when faced with any mounting medium which is not a solution of a resin in a hydrocarbon. As a matter of fact, most simple objects, such as the scales of fish and animal hairs, may be mounted more readily in aqueous media than in resinous ones. The actual process of mounting is so simple that it is regarded with distrust by those who have come to believe that only through complexity can good results be produced. With these media one merely takes the object to be mounted, places it in the drop of mountant on the slide, and presses a coverslip on top. This process, moreover, is not confined to relatively hard objects but may be applied to many protozoa and other small invertebrates. Small invertebrates do not always make satisfactory permanent mounts by this method, for they ultimately reach a refractive index identical with that of the mountants and thus vanish. A temporary mount of a paramecium in one of these media, however, will show the internal structure better than will the average stained mount, while it will also give a clearer indication of what the living object looked like. The most common objects to be mounted by this method are small arthropods; a description of the preparation of a mite in one of these media is given in the second section of this book.

Finishing Slides in Gum Media. Slides may be left exactly as they are prepared, but this will give a rather clumsy appearance since some of the mountant will exude from under the coverslip. This exudate may be removed by washing with warm water, but it will be some time after this process before the gum at the edges of the coverslip is dried. Moreover, no mounting medium containing glycerin can fail to absorb moisture from the air on humid days and to lose it on dry days, so that it is usually better to finish the slide by applying a ring of varnish around the outside.

Preparation of Thick Objects as Wholemounts. Large objects do not usually make good preparations in gum media because they take a very long time to become transparent. It is better, therefore, to mount them in resinous media in the manner about to be described; but, before doing so, it is necessary to explain some of the methods whereby a coverslip may be held in place over a relatively large form. It is obvious that unless support is provided for the coverslip the latter is bound to tip to one side or the other and thus make the mount relatively useless.

A good method of supporting a coverslip over a large object is to secure some sheets of celluloid of various thicknesses and cut these into little squares of about 1 mm. on a side. A drop of the mountant is placed on a slide, the object placed in the mountant, and three of these little squares spaced equally around the edge of it before the coverslip is applied. These squares may be made of any desired thickness, so that one may accommodate objects of varying size. This method, however, is not very satisfactory for objects which are much more than 0.5 mm. thick, since the wide rim left at the edge of the cover causes the mountant to dry out very rapidly. One is forced continually to add additional mountant from the edges.

The alternative is to provide a little box, known as a "cell," and to cement this to the slide before mounting the object in it. Cells are available in a great variety of materials, and the method of their use is described in some detail in Gray's *Microtechnique.* For elementary purposes the best cells which can be used are undoubtedly stamped from sheet tin or pewter and may be obtained in thicknesses varying from ½ to about 1½ mm. They should be cemented to the slide with some cement which is not soluble in balsam. The best general-purpose adhesive is "gold size"; reference should be made to the source cited above for details of this material and the method of its application. Glass cells are available from biological supply houses. Although they look very attractive, there is no real advantage in their use since the object is usually studied by transmitted light.

Mounting in Resinous Media. Resinous media are used for wholemounts not only because they permit the mounting of stained objects but more particularly because they impart to the specimen a great degree of transparency. This transparency comes from the increase in the index of refraction of the specimen when it is completely impregnated with the resin. These resins, however, are not miscible with water, so that one is forced first to remove the water (dehydration) and then to replace the dehydrant with some material (clearing agent) with which the resin itself is miscible. Before these operations are conducted, the specimen must be killed and hardened (fixed), and it is customary to stain the specimen in order to bring out those internal structures which would become invisible, were they not colored, through the increase in transparency. All of the following operations, therefore, must be executed and will be discussed in turn.

1. Narcotizing and fixing.
2. Staining.

3. Dehydrating.
4. Clearing.
5. Mounting.

Narcotizing and Fixing Specimen. Hard objects, such as small arthropods and hairs, may be dehydrated and mounted directly in resinous media but they are far better prepared in gum media. However, most objects which are mounted in resinous media are too soft to withstand the process of dehydration and clearing without special treatment. Though hardening and fixing agents were once considered as separate entities, they are now usually combined into a solution known as a fixative. The more useful of these solutions are given in Chapter 2. Before dealing with fixatives, it is necessary to point out that very few small animals, when plunged into a fixative, will retain their shape, so that it is necessary first to narcotize them in some solution which will render them incapable of muscular contraction.

Narcotization should always proceed slowly; that is, one should add a small quantity of narcotic at the beginning and increase the quantity later, adding the fixative only after cessation of movement. This is easy to judge in the case of motile forms, which may be presumed to be narcotized shortly after they have fallen to the bottom of the container, but in the case of sessile forms it is necessary to use a fine probe, preferably a hair, to determine the end point of narcotization. Some recommended narcotic mixtures are given at the end of Chapter 2. The following suggestions for various types of invertebrates should be used by the student as a basis for further experiment.

NONCONTRACTILE PROTOZOA. These do not require narcotization and may be fixed directly in a chrome-acetic mixture.

INDIVIDUAL CONTRACTILE PROTOZOA. These are very difficult to handle. Individual rhizopods, such as ameba, are best fixed to a coverslip in the following manner. Take a clean coverslip and smear on it a very slight quantity of fresh egg white. Place each individual protozoan in the center of the coverslip and allow it to expand. While this is going on, fit a flask or kettle with a cork through which is inserted a glass tube. The outer end of the tube should be drawn to a fairly fine point. Boil the water in the flask to produce a jet of steam. As soon as the animal is satisfactorily expanded, pick up the coverslip very gently and pass the underside momentarily through the jet of steam. This instantly hardens the protozoan in position and at the same time cements it to the coverslip through the coagulation of the egg white. Then transfer the coverslip to any standard fixative solution for a few minutes before washing and storing in alcohol.

COELENTERATA. Hydroids are usually narcotized with menthol, although the author prefers his own mixture (p. 14) for the purpose, and fixed in a hot mercuric-acetic mixture.

PLATYHELMINTHES. Some of the smaller fresh-water Turbellaria (e.g., Vortex, Microstomum) may be narcotized satisfactorily by adding small quantities of 2 per cent chloral hydrate to the water in which they are swimming. Another good technique is to isolate the forms in a watch glass of water and place the watch glass under a bell jar together with a small beaker of ether. The ether vapor dissolves in the water and narcotizes these forms excellently. A detailed account of the method of handling the liver fluke is given on page 95 and may be employed satisfactorily for other parasitic flatworms.

ANNELIDA. Small marine free-living Polychaeta make excellent wholemounts and do not usually require narcotization before killing. However, they should be stranded on a slide, and a very small quantity of the fixative should be dropped on them, so that they die in the flat condition which makes subsequent mounting possible. Much more realistic mounts are obtained by this means than if the animals are laboriously straightened before fixing, because they usually contract into the sinuous wave which they show when swimming. There seems to be no certain method of fixing the Nereids with their jaws protruding. One has to rely on chance to obtain one in this condition.

FRESH-WATER OLIGOCHAETES. These are best narcotized with chloroform, either by adding small quantities of a saturated solution of chloroform in water or by placing them in a small quantity of water under a bell jar in which an atmosphere of chloroform vapor is maintained. Leeches are rather difficult to handle, and the author has had greatest success by placing them in a fairly large quantity of water to which is added, from time to time, small quantities of a solution of magnesium sulfate. As soon as the leeches have fallen to the bottom, much larger quantities of magnesium sulfate can be added, which will leave the leeches, in a short time, in a perfectly relaxed but not expanded condition. Then they should be flattened between two slides and fixed in Zenker's fluid. After the specimens have been fixed sufficiently long to hold their shape when the glass plates are removed, they are transferred for a couple of days to fresh fixative and then washed in running water overnight.

BRYOZOA. Marine Bryozoa may be narcotized without the least difficulty by sprinkling menthol on the surface of the water containing them. Subsequent fixation is best in some chrome-acetic mixture. It is usually recommended that fresh-water Bryozoa be narcotized in a cocaine solution, but the author has found menthol just as good and very much easier to use. Fresh-water Bryozoa should be fixed directly in 4 per cent formaldehyde since they shrink badly in any other fixative.

ARTHROPODS. Wholemounts of most small arthropods are better made in gum media.

Choice of a Stain. Whatever method of narcotization and fixation has been employed, the specimens, which are to be mounted, are washed free of fixative and accumulated either in water or 70 per cent alcohol.

SMALL INVERTEBRATES AND INVERTEBRATE LARVAE. These are best stained in carmine by the indirect process; that is, by overstaining and subsequent differentiation in acid alcohol. For most specimens the author prefers Grenacher's alcoholic borax carmine.

LARGER INVERTEBRATE SPECIMENS. Larger specimens are better stained by the direct process; that is, exposed for a considerable length of time to a very weak solution of stain and not differentiated.

VERTEBRATE EMBRYOS. These seem to stain more satisfactorily in hematoxylin than in carmine solutions. The author's preference is the formula of Carazzi. Detailed instructions for the use of this stain on a chicken embryo are given on page 89.

PLANT MATERIALS. Plant specimens, since they often consist of only one or, at the most, two layers of cells are easier to stain than are zoological specimens. The nuclei may be stained either with safranin or with an iron hematoxylin technique which in zoological procedures is rigorously confined to sections. A contrasting plasma stain may be used after the nuclei have been well differentiated.

Dehydration. The specimens, plant or animal, stained or unstained, are accumulated either in distilled water or in 70 per cent alcohol according to the treatment which they have had. It is necessary to remove the water from them before they can be mounted in any resinous medium. Ethyl alcohol is widely used as a dehydrant, and, at least in the preparation of wholemounts, only its unavailability should make any substitute necessary. Where substitution is necessary, acetone or methyl alcohol, in that order of preference, may be used. Both have the disadvantage of being more volatile than ethyl alcohol and, therefore, requiring more care in handling.

Dehydration is carried out by soaking the specimen in gradually increasing strengths of alcohol; it is conventional to employ 30 per cent, 50 per cent, 70 per cent, 90 per cent, 95 per cent, and absolute alcohol. The author prefers to omit from this series, unless the object is very delicate, both the 30 per cent and the 50 per cent alcohol, thus starting with direct transfer from water to 70 per cent alcohol. The only difficulty likely to be met in dehydration is in the handling of small specimens because, if they are in specimen tubes, it is almost impossible to transfer them from one to the other without carrying over too much weak alcohol. The author has long since abandoned tubes in favor of the device seen in Fig. 9. This is a short length of glass tube, open at both ends, which has a small piece of bolting silk or other fine cloth tied across the lower end. The specimens are placed in the tubes which, as may be seen in the illustration, are transferred from one stender dish to another with a minimum chance of contamination. These tubes are commercially available in England, but in America must be imported or homemade.

There is no means of judging when dehydration is complete save to attempt to clear the object. It is unwise to believe the label on an open bottle or jar if it says "absolute alcohol" because this reagent is hygroscopic and rapidly absorbs water in the air. Therefore, one should keep a quantity of anhydrous copper sulfate at the bottom of the absolute-alcohol bottle and cease to regard the alcohol as absolute when the salt starts turning from white to blue. More wholemounts are ruined by being imperfectly dehydrated than by any other method. Even the smallest specimen should have at least 24 hours in absolute alcohol before any attempt is made to clear it.

Choice of a Clearing Agent. A clearing agent must be some substance which is miscible both with absolute alcohol and with the resinous medium which has been selected for mounting. The ideal substances for this purpose are essential oils. They impart just as much transparency to the specimen as does the resin used for mounting, so that one has, as it were, a preview of the finished specimen. The use of benzene, which is recommended for preparation of paraffin sections, has started to spread into the preparation of wholemounts. In the author's opinion, it is utterly worthless for this purpose. It has a relatively low index of refraction, so that one cannot tell, if one endeavors to use it, whether or not the slight cloudiness of the specimen is due to imperfect dehydration until after the specimen has been mounted in balsam.

The first choice is terpineol ("synthetic oil of lilac") which has advantages possessed by no other oil. It is readily miscible with 90 per cent alcohol, so that it will remove from the specimen any traces of water which may remain in it through faulty dehydration, and it does not make specimens brittle. It also has a very slight and rather pleasant odor. Clove oil is the most widely recommended essential oil for the preparation of wholemounts. It has only two disadvantages: its violent odor and the fact that objects placed in it are rendered brittle. If a small arthropod is cleared in clove oil, it is almost impossible to get the animal into a wholemount without breaking off some appendages. Clove oil, however, is miscible with 90 per cent alcohol.

Mounting Specimens in Balsam. Nothing is easier than to mount a specimen in balsam, provided that it has been perfectly dehydrated and cleared.

A properly made wholemount should be glass-clear, but it will not be clear in balsam unless it is clear in terpineol or clove oil. Not more than one in a thousand wholemounts which one sees has this vitreous appearance. The worker who is accustomed to looking at rather cloudy wholemounts should take the trouble to dehydrate a specimen thoroughly, to remove the whole of the dehydrating agent with a clearing agent, and then to mount the specimen properly in balsam.

Therefore the first step, in making a mount in Canada balsam, for instance, is to make quite certain that the specimen in its essential oil is glass-clear. The second step is to make certain that one has natural Canada balsam and not dried balsam which has been dissolved in xylene. Solutions of dried balsam in hydrocarbons are meant for mounting sections and, for this purpose, are superior to the natural balsam. Natural balsam is, however, just as preferable for wholemounts and is just as easy to obtain. If it is found to be too thick for ready use, it may be warmed gently to the desired consistency. A single small specimen is mounted by placing it in a drop of balsam on a slide and then lowering a coverslip horizontally (Fig. 10) until the central portion touches the drop. The coverslip is released and pressed very gently until it just touches the top of the object. By this means it is possible to retain the object in the center of the coverslip and also, if one is using natural balsam which does not shrink in drying, to avoid using cells for any but the largest objects. Unfortunately most people are accustomed to mounting sections in thin balsam by the technique shown in Fig. 11; that is, by touching one edge of the coverslip to the drop and then lowering it from one side. The objection to this is that the balsam, as is seen in Fig. 11, immediately runs into the angle of the coverslip, taking the object with it, and it is difficult to lower the coverslip in such a way that the object is left in the center. If one is mounting specimens or deep objects in a cell in which a cavity has been ground, it is desirable to hold the coverslip in place with a clip while the balsam is hardening. This process is seen in Fig. 12; the type of clip there shown is made of phosphor bronze wire and is far superior to any other type.

The description above presumes that one is using natural Canada balsam, unquestionably the best resinous medium in which to prepare wholemounts. If one is using a solution of dried balsam in xylene, a very different technique will have to be adopted. In the first place, most of these solutions are so thin that it is almost impossible to apply the coverslip as shown in Fig. 10, and one is forced to adopt the technique shown in Fig. 11. This difficulty may be avoided by placing the object on the slide, putting a drop of the medium over the object, and then setting the slide in a desiccator until most of the solvent

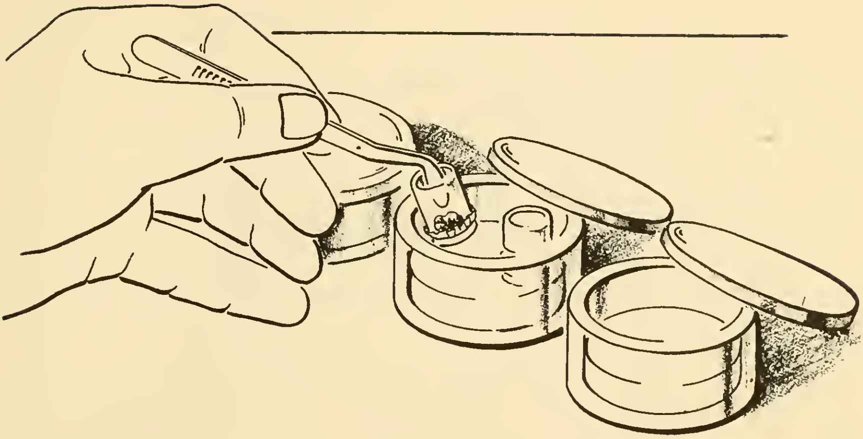


FIG. 9. Transferring objects between reagents with cloth-bottomed tubes.

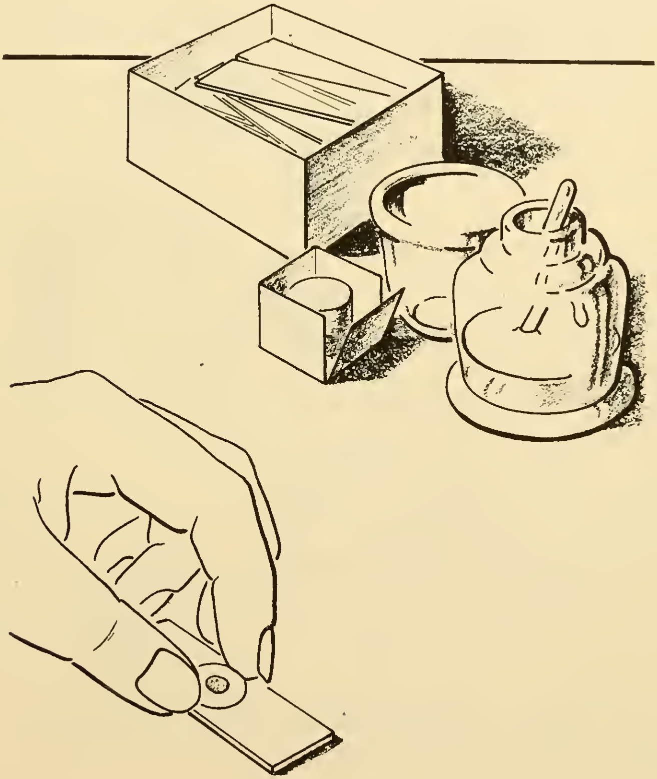


FIG. 10. Applying coverslip to balsam wholemount.

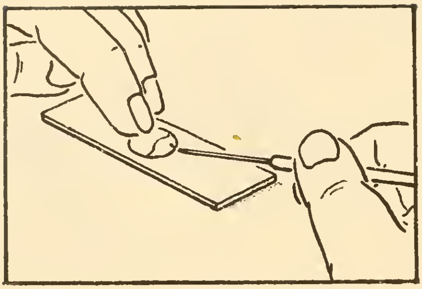


FIG. 11. Wrong way to apply coverslip to balsam wholemount.

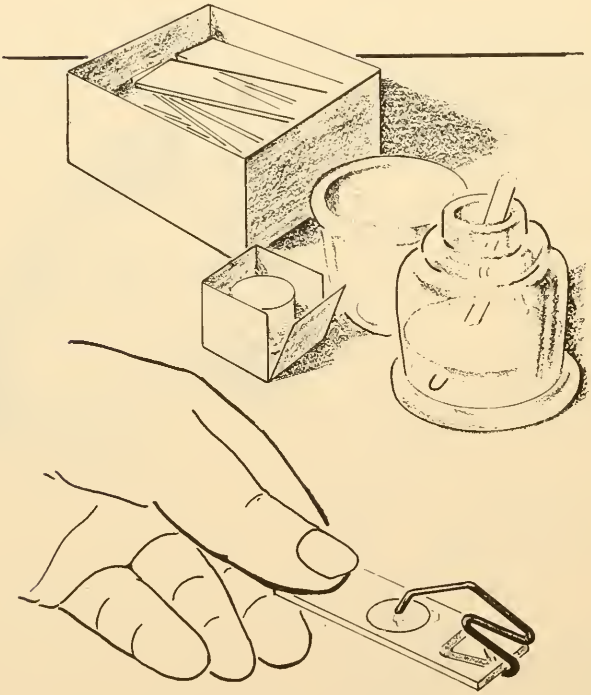


FIG. 12. Balsam wholemount ready for drying.

has evaporated. A second layer is placed on top to build up a large drop or, rather, a thick coat of varnish over the specimen. A coverslip is applied, and the slide is warmed until the resin becomes fluid.

The best use for solutions of balsam in making wholemounts is in dealing with a very large number of objects. The method in this case is to transfer the objects from their clearing medium to a tube or dish of the solution of balsam in whatever hydrocarbon has been selected and then permit the hydrocarbon to evaporate. When the balsam which remains has reached a good consistency for mounting, take each specimen, together with a drop of balsam, place it on a slide, and add a coverslip. By this method large numbers of slides can be made in a short time. It is not necessary to use solutions of dried balsam, and the author prefers, for this purpose, to dilute natural balsam with benzene. Mounting large objects in a deep cell in Canada balsam is not to be recommended because the balsam becomes yellow with age and, when in thick layers, tends to obscure the specimen. A wholemount of a 96-hour chicken embryo, for example, is of extremely doubtful value, but if it has to be made it is best first to impregnate it thoroughly with a fairly thin dilution of natural balsam. It is then placed in the cell, piling the solution up on top, and left in a desiccator. The cell is refilled as the evaporation diminishes the contents; when completely filled with solvent-free balsam, it is warmed on a hot table. The coverslip is applied directly.

Finishing Balsam Mounts. If a mount has been made correctly with natural balsam, and if the size of the drop has been estimated correctly, no finishing is required since no balsam will overflow the edges of the coverslip. Natural balsam is very thick when cold, so that the coverslip will not become displaced if the mount is handled before the balsam is fully hard. The hardening may be aided by heat, but one must be careful in heating thick mounts, particularly where the coverslip is not supported by a cell, that the liquefaction of the balsam does not cause the coverslip to tip sideways. Despite the fact that drying time is sometimes prolonged, natural balsam should always be used for these thick mounts because, if a solution of dried balsam and xylene is employed, the evaporation of the solution will cause huge air bubbles to be drawn under the coverslip. When it is sufficiently hard, the slide should be cleaned, first, by chipping off any excess balsam with a knife and, secondly, by wiping away any chips with a rag moistened in 90 per cent alcohol. This will leave a whitish film over the surface of the slide, which may then be removed with a warm soap solution. The slide should be polished before being labeled.

With regard to labeling, it may be pointed out that no power on earth will persuade gum arabic, customarily used for attaching labels, to adhere to a greasy or oily slide. Therefore, the portion of the slide to which the label is to be attached should be cleaned more carefully than any other. The author prefers to moisten both sides of the label, press it firmly to the glass, and write on it only after it is dry.

7

Making Smears

The last chapter was concerned with the preparation of microscope slides from whole objects preserved as nearly as possible in their natural shape; the next chapter will be concerned with the preparation of thin slices of objects or "sections." Between the extremes of a whole object and a thin slice, there is the type of preparation which is discussed in this chapter. This is the "smear," which is exactly what its name indicates; that is, it is prepared by smearing some substance on a clean glass slide where it may be fixed, stained, and mounted. Three operations are necessary in the preparation of smears of fluids: (1) The smearing of the material itself into a layer of the required thickness; (2) the fixing of this layer both to insure its adherence to the slide and to make sure that the contained cells remain in their normal shape; and (3) the staining and mounting of the fixed smear. Each of these operations will be discussed successively.

Preparation of the Smear. The first thing to do in the preparation of a smear is to make sure that there are some chemically clean slides available. Only materials containing large quantities of protein, such as blood, will adhere to slides that are not perfectly clean. Any method may be used for cleaning slides. For this particular purpose, however, the author prefers to take any household "scouring powder," which consists of a soft abrasive together with some detergent agent. The powder is made into a thin cream with water. Each slide is then dipped into the cream and put in a rack to dry. As soon as it has dried, the slide may be returned to a box, preferably separated from the next slide with a thin paper insert. Since slides are commonly sold with paper separators, they may be stored with the separators in the original box.

Two or three hundred slides may be prepared easily and quickly in this manner and stored for future use. When a slide is needed, the white powder is polished from the surface with a clean linen or silk cloth. Smears often have to be made at unexpected moments, so that it is a great convenience to have slides at hand which may be rendered fit for use in a few moments.

The actual method of smearing varies greatly according to the material being used. Probably more smears are made of blood than of any other fluid, and the technique for the preparation of these is so well established that it will be described as a type. The material itself either may be taken from the puncture wound directly onto the slide or, as in Fig. 13, removed from the puncture wound with a pipette and transferred to the slide. The drop is placed about a third of an inch from one end of the slide. A second slide, as shown in Fig. 14, is placed on the drop. Capillary attraction, naturally, will distribute the fluid along the edge of the second slide, which is then (Fig. 15) pushed sharply forward until it reaches the end of the bottom slide. The material of which the smear is made is thus dragged out behind the first slide and distributed more or less uniformly on the under slide. A few people still try to conduct the operation in the reverse manner by placing the second slide on top of the first, sloping it at a reverse angle to that shown, and then endeavoring to push rather than drag the material across the lower slide. The objection to this is that it results in crushing cells, although it must be admitted that it frequently results in a more uniform distribution of the material.

The method just described is the standard procedure for producing "thin smears." These are necessary for those fluids, such as vertebrate blood or mammalian seminal fluid, which contain large numbers of objects requiring wide separation for satisfactory study.

There are a number of fluids, however, from which thick smears must be made either because they contain relatively few cells, as in the case of invertebrate blood, or because they contain parasites which are distributed relatively sparsely through the material, as in the case of malarial diagnostic smears. These thick smears are commonly made with the aid of a loop of wire held in a needle holder of the type found in bacteriological laboratories. This loop is dipped into the fluid to be examined. The material is spread with a rotary motion in the center of the slide. This is very similar to the preparation of smears of bacterial material which is described in some detail on page 100.

Fixing Smears. Smears may be fixed by drying, by alcohol, or in one of the conventional fixatives. When a smear is to be fixed only by drying, it is dried by waving it in the air, as soon as it has been made, and then set aside for subsequent treatment. This procedure is excellent in the case of objects, such as bacteria or erythrocytes, which do not change their shape after drying; or for materials, such as white blood corpuscles, which it is not desired to preserve in their normal shape. No other object, however, can be considered satisfactory unless it has been fixed. The simplest method of doing this is to pass the smear, just as it is drying, through a jet of steam. This technique has already been described (see p. 42) for mounting amebas and need not be repeated here.

All other smears should be fixed before they are dried, and it is something of a problem to fix them without removing the material from the slide. It is obvious that if the material is freshly smeared onto a glass slide and then dropped into a fixative of some kind or another, it will be likely to be washed off. The logical solution to the problem is to use a fixative in a vapor phase,

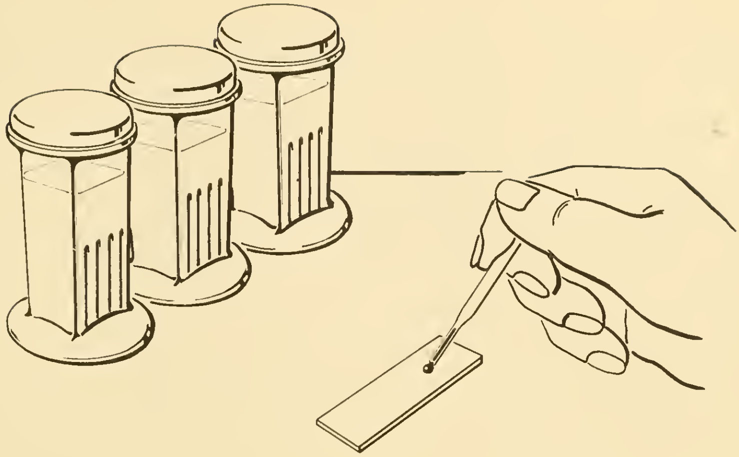


FIG. 13. Making a smear preparation. *a. Place the drop about an inch from the slide.*

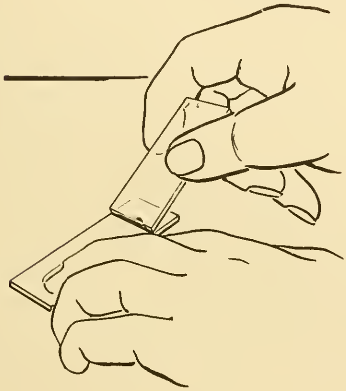


FIG. 14. Making a smear preparation—*(continued). b. Apply a second slide just in front of the drop.*

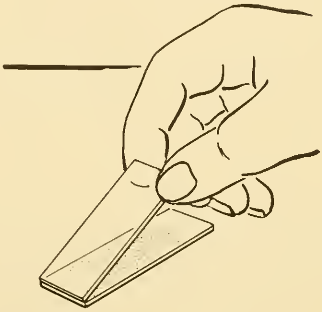


FIG. 15. Making a smear preparation — *(continued). c. Push the slide smoothly forward to spread the smear.*

and nothing is better for this purpose than osmic acid. To use this material, take a petri dish and place in it a couple of thin glass rods sufficiently far apart to permit the slide to rest on them without the smear touching them. Then place a drop or two of a solution of osmic acid, usually of 2 per cent strength, in the bottom of the petri dish and replace the cover. It must be emphasized that osmic acid fixes the mucous membrane of the nose and throat just as readily as it does a smear, and every caution must be taken to avoid inhaling the vapors of this material. As soon as the smear is made *and before it has time to dry,* it is placed face down across the two glass rods, so that it is exposed to the vapor but not to the liquid. The cover is then replaced on the petri dish, and the slide left in place for 3 to 4 minutes, in the case of a thin smear, or 5 to 10 minutes in the case of a thick one. Then it is transferred to distilled water to await staining.

It occasionally happens that one must fix a slide in one of the conventional fluid fixatives. This is done with the same petri dish and glass rod setup as is used for vapor fixation, but in this instance the fixative is carefully poured into the petri dish, which must be level, until it has reached such a depth that, when the slide is laid across the glass rods, the under side of the slide with the smear on it is in contact with the fluid while the upper part is free from fluid. If the smear is reasonably thin and is laid carefully in place, it usually will not become detached.

Staining Smears. Blood smears are stained so universally with one or another of the methylene blue eosinate mixtures (see p. 27) that it comes as something of a surprise to most people to learn that any stain which is suitable for sections may also be employed for smears. The advantage of these mixtures for blood films is that the solvent methyl alcohol acts as a fixative, so that the films, in effect, are stained and fixed in the same operation. Where a blood smear is to be used for diagnostic purposes, these techniques are excellent since the appearance of the various types of white corpuscles under this treatment is known to every technician. For materials other than blood, there is no limit to the type of staining which may be employed, although it must be remembered that these very thin films require a stain of considerable intensity if the finer structures are to be seen.

8

Making Sections

Nature of the Process. A section is a thin slice cut from biological materials with a view to studying either the cells themselves or their arrangement, neither of which can be made out from a wholemount.

Though sections may be cut at any angle, they are usually taken through any one of three planes (see Fig. 16) which are known as "transverse," "sagittal," and "frontal" planes. The purpose of this orientation of the material is to enable one to realize better the structure of the whole from an examination of the section. Theoretically, to produce a section, it is necessary only to take a sharp knife and cut a thin slice from the object under examination. Very few materials, however, are suitable for this, and this procedure does not produce sections of the same thickness. Therefore, it is customary to employ an instrument known as a "microtome" which is a device for advancing a block of tissue a given amount, cutting a slice from it, readvancing it the same amount, and repeating the process.

Another objection to the mere cutting of slices from an object is the nature of biological specimens themselves. Very few of these are stiff enough to withstand the action of the knife without bending, and many contain cavities which would be crushed out of recognition as the section was taken. It is customary, therefore, for most biological work to surround and support the object to be cut with some material which will impregnate its whole substance. The medium most commonly used to support structures is wax. The technique for cutting wax sections is described on page 66. There are, however, a number of materials which may be cut without either complicated microtomes or the support of impregnating substances. Sections which are so cut are known as "free" or "freehand" sections.

Microtome for Free Sections. Even if the material itself is of the correct consistency to withstand the action of the knife, it is still necessary to have some mechanism which will permit one to produce sections of known thickness. The type of microtome usually employed in hand sectioning is shown in Fig. 17 and consists essentially of a disc, usually of highly polished plate glass, supported on a cylinder which is gripped in the hand. For holding specimens within this cylinder there is a mechanism which terminates at its lower end on a micrometer screw. When this screw is turned, therefore, the object in the holder is pushed above the surface of the glass plate. The collar of the micrometer screw is graduated, sometimes in thousandths of an inch but more usually in hundredths of a millimeter. The unit commonly employed to

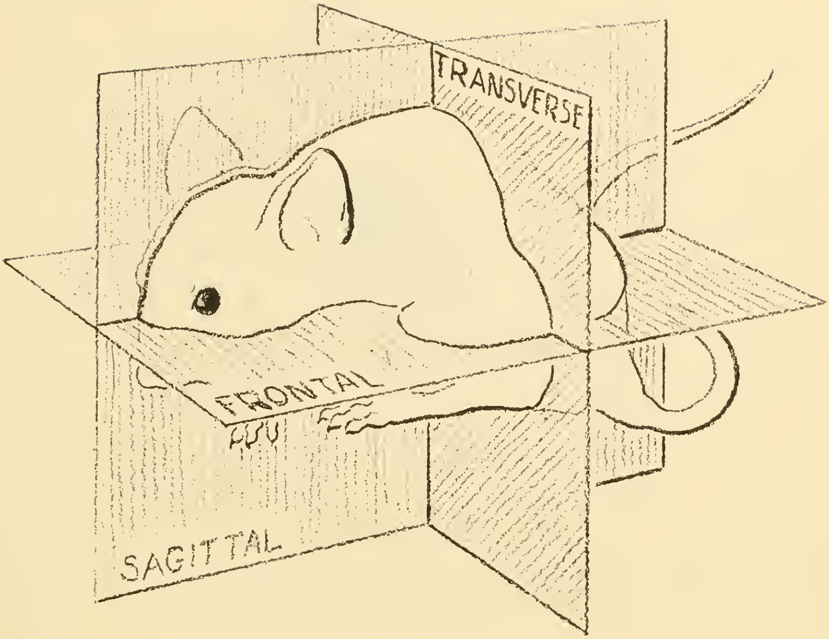

TRANSVERSE
FRONTAL
SAGITTAL

FIG. 16. Standard section planes.

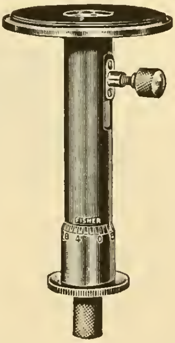


FIG. 17. Hand microtome.

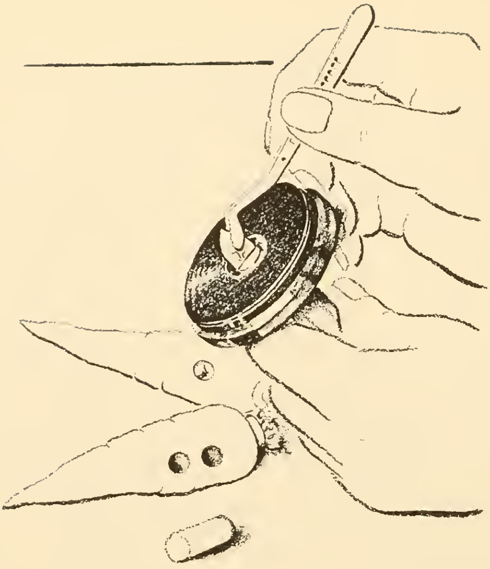


FIG. 18. Inserting a leaf into a split cylinder of carrot.

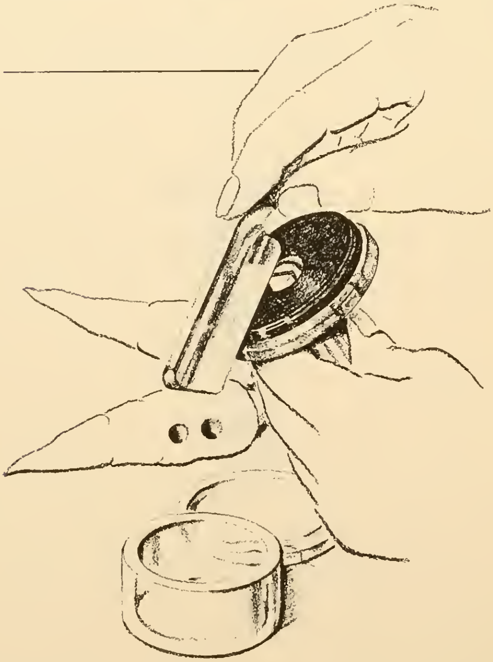


FIG. 19. Cutting a hand section. *The razor is drawn across the plate with gentle pressure and the section then washed into a stender dish.*

describe the thickness of a section is a "micron" which is one thousandth of a millimeter, but hand sections are rarely cut less than 10 microns thick and are usually better at two or three times this.

Methods of Holding Material. The material, although it may be suitable for cutting, is rarely of a size and shape which may be gripped in the holder of the hand microtome without additional support. Therefore, it must be held in some substance which itself will cut readily and which may be shaped easily to support the material to be cut. It is perfectly possible to embed the material in wax before cutting a hand section, but if one is to go to this amount of trouble it is usually better to employ a complex microtome of the type described on page 61. Vegetable tissues are generally used to support objects for hand sectioning. The two best known are "elder pith" and carrots. Elder pith has the advantage that it may be stored indefinitely and cuts with a clean crisp action. Unfortunately, the pith of the American elder *(Sambucus canadensis)* does not appear to be as suitable for the purpose as the pith of the European elder *(S. nigra)*. This difference between the two species may account for the disfavor in which elder pith is held in the United States, for in the author's experience it is far more convenient than the carrot. The disadvantage of the carrot is that it must be absolutely fresh and, even if it is kept in water overnight, it loses much of that crispness which is necessary for the production of a good section.

Almost all hand sections are cut from botanical material, the majority of them from leaves or stems. To support a leaf, merely cut a cylinder of the right diameter to fit in the microtome from either elder pith or carrot, split it down the middle, insert the leaf (Fig. 18), and then tighten the holder. Stems, however, cannot be held by this means, so that one must obtain a hollow cylinder, having an outer diameter convenient to the microtome being employed and an inner diameter slightly less than that of the stem to be gripped. This hollow cylinder is split, the stem inserted, and the section cut (Fig. 19). Of course, a few substances, such as cork or stiff plant stems, may be cut without any other support; these are, however, in the minority.

Hardening and Fixing Materials for Cutting. Many objects which are in themselves unsuitable for sectioning by hand may be rendered more suitable if they are fixed and hardened in some chemical reagent. If, however, one is to go to the trouble of hardening and fixing material in a formula designed to preserve the structure of the cells, it is usually worth while going to the additional trouble of embedding the material and cutting paraffin sections. It is generally sufficient for material, which is to be hand sectioned, to be preserved in 90 per cent alcohol, a process which is equally applicable to the stems and leaves of botanical specimens or to the very few animal materials, such as cartilage, which are suitable for the production of hand sections.

Staining and Mounting Hand Sections. Sections are taken from the knife as individual objects and are accumulated in a dish of some preservative, usually 70 per cent alcohol. They should be treated as wholemounts rather than as sections. That is, either they may be mounted directly in gum media or they may be stained and mounted in resinous media in the manner described in Chapter 6.

Paraffin Sections. Preparation of paraffin sections is quite a complex operation and involves the following stages:

1. Fixation of the material.
2. Dehydration in order that the material may be impregnated with a fluid capable of dissolving wax.
3. Removal of the dehydrating agent with a material solvent of, or miscible with, molten wax.
4. Soaking the cleared specimen in molten wax long enough to insure that it will become completely impregnated.
5. Casting the now impregnated specimen into a rectangular block of wax.
6. Attaching this block of wax to some holder which itself may be inserted into a suitable microtome.
7. Actual cutting of the sections of the block into ribbons.
8. Placing these ribbons on a glass slide in such a manner that they will lie flat and that the contained section will be adherent after the wax has been dissolved.
9. Removal of the wax solvent.
10. Staining and mounting.

Each of these operations will be dealt with in due order. In the second section of this book there are a series of examples which describe in detail the application of these principles to actual preparations.

Choice of Fixative. The methods described in Chapter 6 for the fixation of objects for wholemounting can be used equally well if these objects are to be sectioned. The selection of the fixative for blocks of tissue, however, is based more on the nature of the detail which is to be preserved. In general, it may be said that strongly acid fixatives are best where nuclear detail is required. The selection of fixatives and several hundred formulae for the solutions involved, are given in Gray's *Microtechnique* to which reference should be made by the student seeking special information. For elementary histological preparations, the fluids of Zenker, Gilson, or Petrunkewitsch, the formulae for which are given in Chapter 2, are all excellent.

Dehydrating. The classic method of dehydration is to soak the object in a graded series of alcohols, usually 10 or 15 per cent apart. Dehydration through gradually increasing strengths of alcohol may be vital when one is dealing with delicate objects containing easily collapsible cavities, such as chick and pig embryos, but a block of tissue may be taken from water to 96 per cent alcohol without any appreciable damage. Even if one uses increasing strengths of alcohol, the series normally in employment at the present time is by no means satisfactory. It is customary, for example, to pass the object from water to 30 per cent alcohol at one end of the series and to pass it from 85 per cent to 96 per cent at the other end of the series. An intelligently graded series for delicate objects should run from water to 15 per cent alcohol to 40 per cent alcohol to 75 per cent alcohol to 96 per cent alcohol rather than through the conventionally spaced gradations. This is not at all in accordance with the recommendations in most textbooks but is based on the author's experience over a long period of time. In using this classic method of dehydration, it is not necessary to confine the technique to ethyl alcohol. Methyl alcohol or acetone will dehydrate just as effectively although they are more volatile.

The substitution of a solvent which is miscible both with water and molten wax for a straight dehydrating agent is in vogue today. The best-known of these solvents is dioxane, though *n*-butyl alcohol has also been recommended. The author is not completely satisfied with these methods because, although the solvents involved are excellent dehydrating agents, they are relatively poor solvents of paraffin and frequently occasion great shrinkage of delicate objects in the final transition between the solvent and the wax. For such objects as the routine examination of the tissue blocks in a pathological laboratory or the sectioning of relatively sturdy plant materials, they may justifiably be employed. However, for sections in which structures are to be retained intact for subsequent research, it is to be recommended most strongly that the standard routine of passing from a dehydrating to a clearing agent be followed.

Clearing. The choice of a clearing agent in section cutting is of far more importance than the choice of a dehydrant, since there is not the slightest doubt that prolonged immersion in xylene leads to a hardening of the tissue with subsequent difficulty in sectioning. Benzene is much to be preferred for most objects.

It is still recommended occasionally that essential oils, such as cedar oil, be used for clearing objects for embedding. There is no justification for this unless it is vital that the object be rendered transparent rather than alcohol free, in order that some feature of its internal anatomy may be oriented in relation to the knife. Essential oils are excellent for wholemounts but they are not readily removed from the specimen by molten wax, so that if they must be used they should always be washed out with a hydrocarbon before the wax bath. Relatively small traces of any essential oil will destroy the excellent cutting properties of any wax mixture and, as the oils are nonvolatile, there is no chance of getting rid of them in the embedding oven.

Choice of an Embedding Medium. It is to be presumed at the present time that no one will endeavor to use a plain paraffin but will use a mixture. If a plain paraffin is preferred, then it is necessary to buy (in the United States by importation) a carefully fractionated and very expensive wax. Ordinary cheap paraffin is a mixture of a great variety of compounds of slightly different melting points; it is essential in the use of pure wax that a wax of a very sharp melting point should be obtained.

The choice of an embedding medium should be dictated less by the nature of the specimen than by the conditions under which it should be cut. If pure paraffin is to be employed, it should be of such a melting point that will give the hardened wax a crisp section at the required room temperature. Since the introduction of any foreign substance automatically lowers the melting point of the wax, it is obviously desirable to use mixtures rather than the pure material. For ordinary routine preparations, the author's preference is for any of the paraffin-rubber-bayberry wax mixtures. The introduction of rubber undoubtedly increases the stickiness of the wax and makes it easier to secure continuous ribbons. Bayberry wax not only prevents the crystallization of the paraffin but also lowers its melting point. Two of the best mixtures are:

HANCE'S RUBBER PARAFFIN:

Stock rubber solution

Cut 20 Gm. of crude rubber into small pieces and dissolve, with constant stirring, in 100 Gm. paraffin heated to smoking.

Embedding wax

Paraffin	100 Gm.
Stock rubber solution	4 Gm.
Beeswax	1 Gm.

MAXWELL'S EMBEDDING WAX:

Paraffin	100 Gm.
Hance's stock rubber solution	4 Gm.
Bayberry wax	7 Gm.
Beeswax	1 Gm.

It is not recommended that these embedding waxes be prepared in the laboratory. There are many proprietary embedding waxes, sold under a variety of trade names, which have essentially the composition of the two mixtures just described.

The Technique of Dehydrating, Clearing, and Embedding. Before passing to the choice of a microtome and the method of using it, it is necessary to discuss briefly the actual operations which are involved in using the dehydrating, clearing, and embedding media selected. The techniques of dehydration and dealcoholization do not differ materially from those used in the preparation of wholemounts which have been described. The whole process, however, could be much simplified if people would only remember that water is heavier than the majority of dehydrating agents and that the majority of dehydrating agents are lighter than most clearing agents. In translating this theory into practice, it must be obvious that the object to be dehydrated should be suspended toward the top of a tall cylinder of dehydrant, in order that the water extracted from it may fall toward the bottom of the vessel, and that an object to be cleared should be held at the bottom of the vessel for the reverse reason. It is, indeed, practically impossible to dehydrate a large object unless it is so suspended. The process of impregnating the tissues with wax has not been discussed previously and will be dealt with fully.

It is obvious that the first prerequisite is some device which will maintain the temperature of the wax at just above its melting point. Most people employ complex thermostatically controlled ovens for this purpose, but the exceedingly simple device shown in Fig. 20 is practical and cheap. As will be seen, this device consists essentially of a series of incandescent electric bulbs held at a distance which may be varied above a series of glass vials. Before the embedding process is started, as many vials as will be required are filled with wax, placed under the reflector, and the current is turned on. After a little while, it will be observed that the absorbed heat has melted the wax. The wax may be melted only at a small surface layer, throughout the entire vial, or, as is required, in the upper two thirds of the vial. If this last is not achieved, the height of the lamp must be varied until after an hour or two each of the vials contains about one third of unmolten opaque wax at the bottom and two thirds of the clear molten material above. Thus, when the object is placed in one of these vials, it will drop until it reaches the solidified layer where it will remain in contact with molten wax at exactly the melting point of the wax. It is obvious that the room in which this operation is to be conducted must be at a fairly constant temperature and must be relatively free of drafts, but only a very large volume of embedding work justifies the purchase of an expensive thermostatically controlled oven. If such an oven is to be purchased, it is highly desirable to avoid one in which the heat is distributed by convection. The oven shown in Fig. 21, in which a circulating fan continuously moves the air and thus maintains a uniform temperature throughout the whole oven, is infinitely to be preferred. It is the high cost of such circulating air ovens which leads the author to believe that much more use should be made of the very simple radiant heat embedding device just discussed.

Assuming that the material has been passed through dehydrating and clearing agents and is now awaiting embedding, there are two main methods by which this may be done. Either the object may be transferred directly to a bath of molten wax, or it may be passed through a graded series of wax-solvent mixtures. The author is strongly in favor of the latter course. Let us suppose that we have selected benzene as the clearing agent, and that the object is in a vial containing a few milliliters of this solvent. Take a block of whatever medium is to be used for embedding and shave a few chips from it with a knife. Add these to the solvent. The chips usually dissolve very slowly and form a thickened layer at the bottom of the tube through which the object to be embedded sinks. The average object will be satisfactory if left overnight. Then place the tube itself in the embedding oven maintained at a temperature somewhat above that of the melting point of the wax and add as many further shavings as can be crammed into the tube. When these are completely melted and a large quantity of the volatile solvent has evaporated, remove the object with a pipette or forceps and place it in a dish of pure wax for an hour or two before transferring it to a second dish of pure wax for the time necessary to secure complete impregnation.

There is no method of forecasting how long an object will take to become completely impregnated with wax. It is very easy to find out (see Table 1) when one has started to cut sections whether or not the impregnation is complete, but there is no basis save experience on which to establish the timing in the different baths of wax. If the object is to be transferred directly from solvent to wax, at least three baths should be employed since nothing is more destructive to a good section than the presence of a small quantity of the clearing agent in the embedding medium. To an absolute beginner seeking a rough guess, it may be said that a block of liver tissue, 3 to 5 mm. in size, will be satisfactorily impregnated with wax after 30 minutes in each of three baths, while a 96-hour chick embryo will require at least two hours in each of three baths for its successful impregnation.

While the object is being impregnated with the wax, it is necessary to decide what type of vessel will be used to cast the final block. This will depend to a far greater extent on the size of the object than on the preference of the worker. Very small objects may be embedded most satisfactorily in ordinary watch glasses (that is to say, ordinary thin-walled watch glasses, not syracuse watch glasses of the laboratory type) or in any other thin-walled glass vessel.

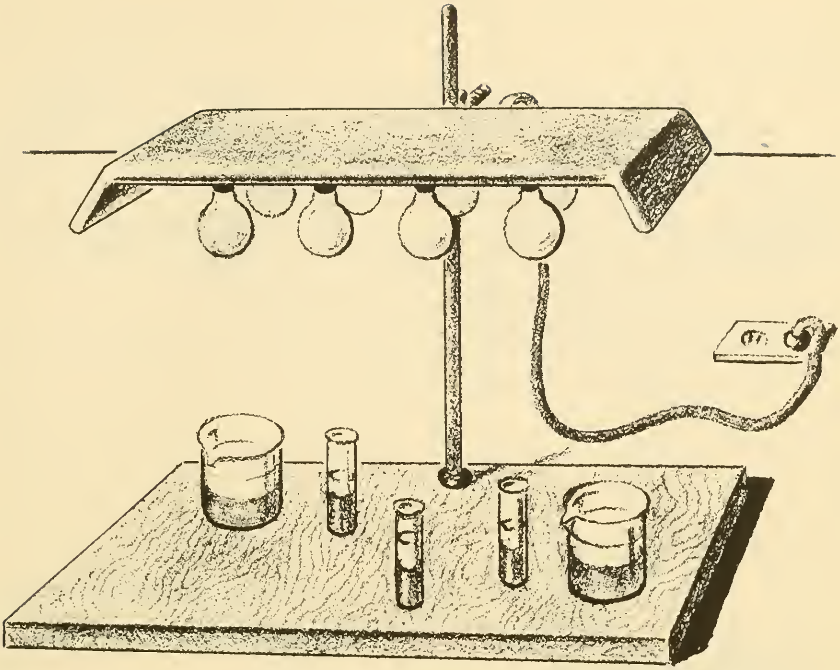


FIG. 20. Simple radiant heat embedding oven. *Height of the hood should be adjusted until the wax is melted for about one-half its depth.*

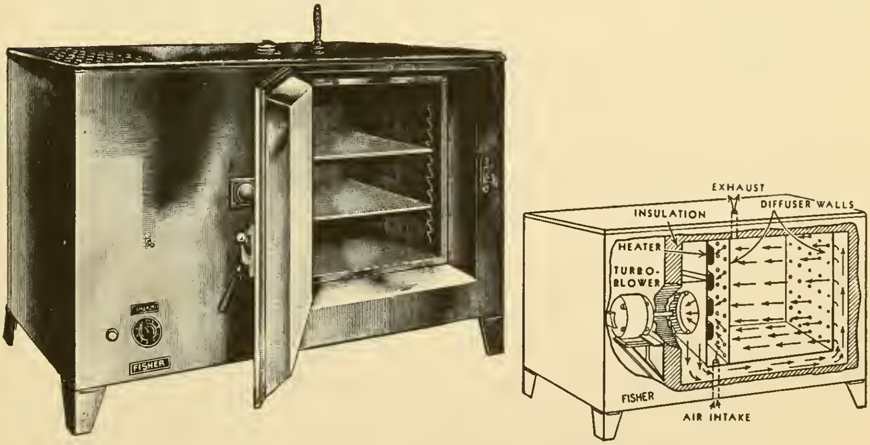

FISHER
EXHAUST
INSULATION
DIFFUSER WALLS
HEATER
TURBO-BLOWER
FISHER
AIR INTAKE

FIG. 21. Circulating air embedding oven.

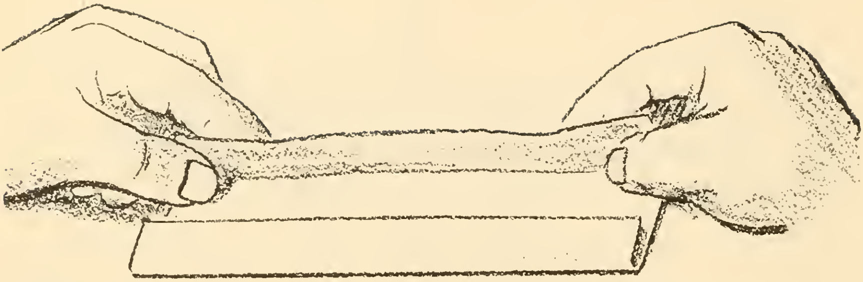


FIG. 22. Folding a cardboard box. *a. The two long edges of a rectangular card are folded to meet in the center.*

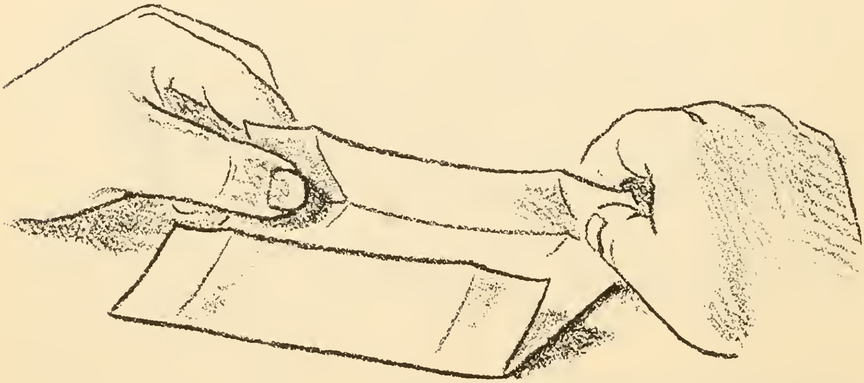


FIG. 23. Folding a cardboard box—*(continued). b. Folds are flattened out and the short edges are folded not quite to the center.*

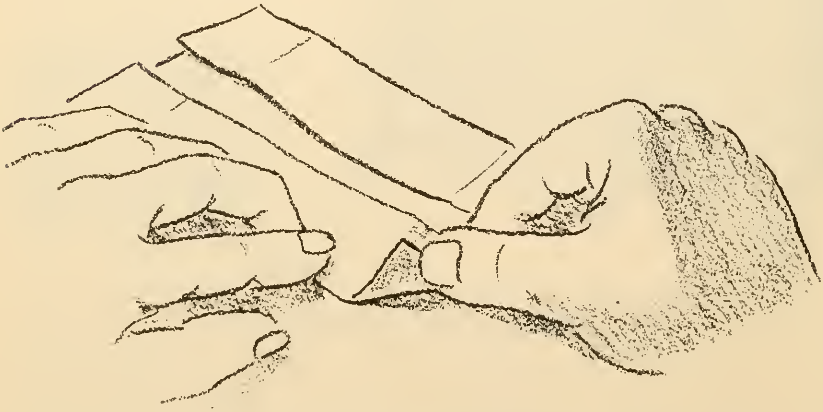


FIG. 24. Folding a cardboard box—*(continued). c. Corners are folded over.*

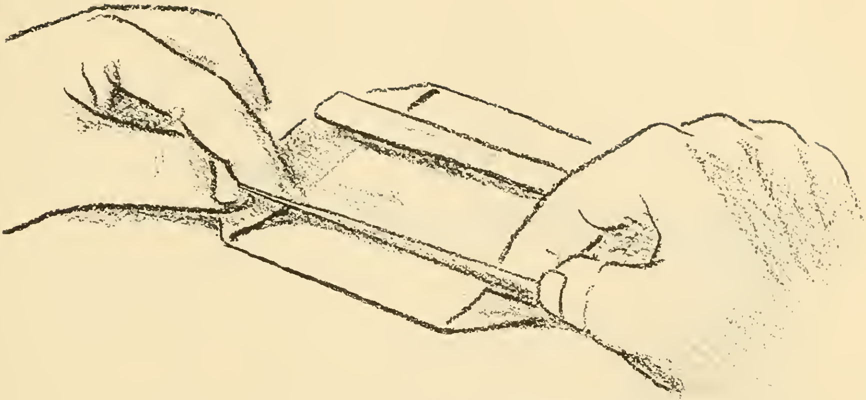


FIG. 25. Folding a cardboard box—*(continued). d. Edge of the fold is folded back over the creased corners.*

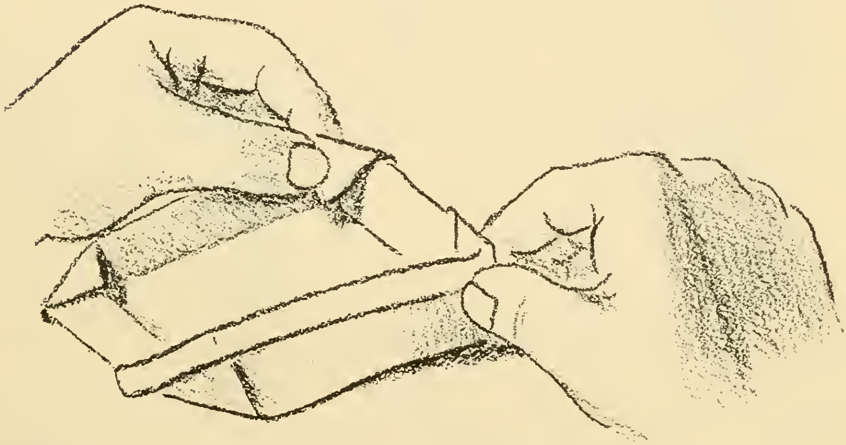


FIG. 26. Folding a cardboard box—*(continued). e. Box is opened and the corners pinched.*

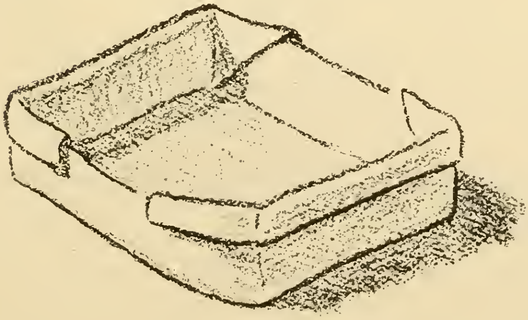


FIG. 27. Folding a cardboard box—*(continued). f. Finished box.*

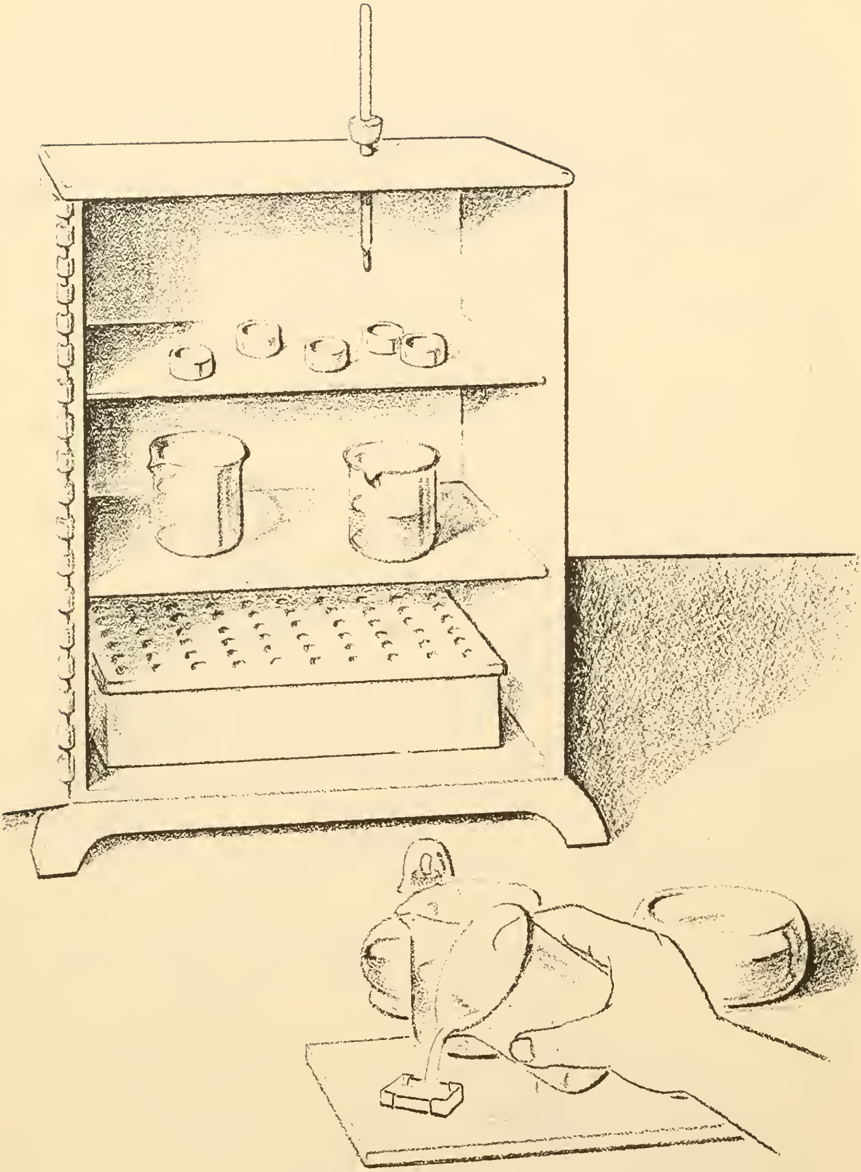


FIG. 28. Filling with wax an embedding box which has been attached with water to a glass slide.

Very large objects are often embedded with the aid of two thick L-shaped pieces of metal which, when fitted together, form a rectangular mold of varying dimensions. The author regards these as very clumsy and would always prefer to prepare a cardboard or paper box than to endeavor to maneuver metal molds which are always getting jarred out of place at the wrong moment. The preparation of a paper or cardboard box is easy; the method preferred by the author is shown in Figs. 22-27.

A rectangular sheet of thin cardboard or stout paper approximately twice as long as it is wide is needed. The area of the floor of the box will be about one third that of the sheet taken, but a little experience will soon show what size sheet to select for the box required. The sheet is laid on a flat surface, and the long sides are folded inward (Fig. 22) until they almost meet in the middle. These folds are well creased with the thumb nail. The sheet of paper is flattened again, and the other two edges (Fig. 23) are folded in the same manner. It is necessary, however, that these folds be much larger than the first folds made. These folds are also well creased with the thumb nail. Then the folded sheet is laid out (Fig. 24), and the corners are folded in the manner shown. Since these end folds are larger than the side folds, there will be an overhanging flap of paper at the top. After all four corners have been folded in this overhanging flap (Fig. 25), the flap is folded back over the triangularly folded corner sections, and this crease particularly firmly pressed with the thumb nail. When this has been done at each end, the box is finished and may be opened out as shown in Fig. 26. It will be found that the corners are not square, but may be squared by pressing with the thumb and forefinger in the manner shown. The finished box is shown in Fig. 27. This box is a great deal larger than that usually employed but has been selected for ease of illustration. Some people prefer to cast themselves a series of rectangular boxes from plaster of Paris. This can be done by any competent craftsman but will not be described.

After the box has been prepared, the actual process of embedding is begun. This is shown in great detail in Figs. 28-31. Before starting, it is necessary to make sure that one has available the following items: (1) A dish of water of sufficient size that the finished block may be immersed in it readily; in the illustration an ordinary laboratory finger bowl is in use, (2) some form of heat, an alcohol lamp being just as effective as a bunsen burner, (3) a large slab of plate glass, and (4) a wide-mouthed, eye-dropper type pipette. The oven employed should contain the object itself in a small container of molten wax, as well as another container of the medium used. It must be emphasized that one cannot impregnate an object with one kind of wax and embed it in another.

The first thing to do is to wet the *underside* of the bottom of the paper box in the water and press it firmly into contact with the plate-glass slab which will both hold it in position and assist in cooling the wax. Then take (Fig. 28) one of the beakers of molten embedding material from the oven and fill the little paper box to the brim. Heat the pipette in the flame to a temperature well above that at which the wax will melt. Use it to pick up the object from its own dish (Fig. 29) and to transfer it to the little paper boat. By the time that this has been done, a layer of hardened wax will have been formed at the bottom of the paper boat, so that the object will rest on the layer of solidified wax with a molten layer above it. It will happen almost invariably that the surface has also cooled, so that a crust of cool wax will have been carried down with the object into the box. It is essential to get rid of this if the wax is to adhere through section cutting. Reheat the pipette; use it to melt the entire surface of the box (Fig. 30) and to maneuver the object into the approximate position in which it is required to lie in the finished block. Then blow on the surface until the wax is sufficiently solidified to enable you to pick up the box carefully and, as shown in Fig. 31, hold it on the surface of the water used for cooling. With most wax media it is desirable to cool the block as rapidly as possible, and it should never be permitted to cool in air. It cannot, however, be pushed under the surface of the water because the molten center is liable to break through the surface crust and thus destroy the block. After holding the box in the position indicated until it is fairly firm throughout, push it under the surface to complete the cooling.

The block may be left in water for any reasonable length of time, but if it is to be stored for days or weeks it is much better kept in a 5 per cent solution of glycerin in 70 per cent alcohol. There seems to be a widespread delusion that, because an object must be perfectly dehydrated before being impregnated with wax, it must subsequently be kept out of contact with fluids. Nothing could be farther from the truth. As will be discussed later, when dealing with the actual technique of sectioning, it is often desirable to expose a portion of the object to be sectioned and leave it under the surface of water for some days in order to get rid of the brittleness which has been imparted through the embedding process. Blocks which have been stored dry for a long period of time should always be soaked in a glycerin-alcohol mixture for at least a day before sectioning.

It is, in any case, undesirable to section a block as soon as it has been made, because it is necessary for successful sectioning that the block be the same temperature throughout. If a block is made in the evening, it is better to take it out of the water and to leave it lying on the bench overnight, in order that the temperature may be stabilized.

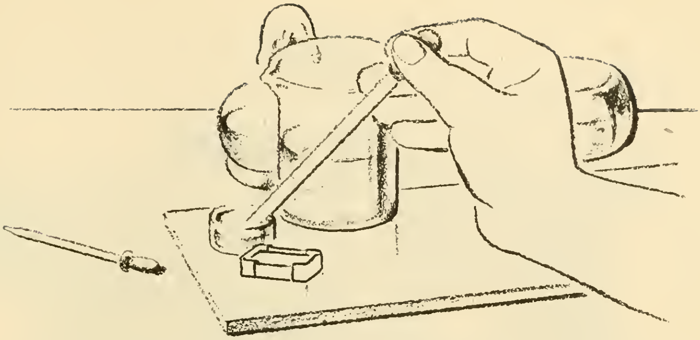


FIG. 29. Transferring the object from the embedding dish to the wax-filled paper box.

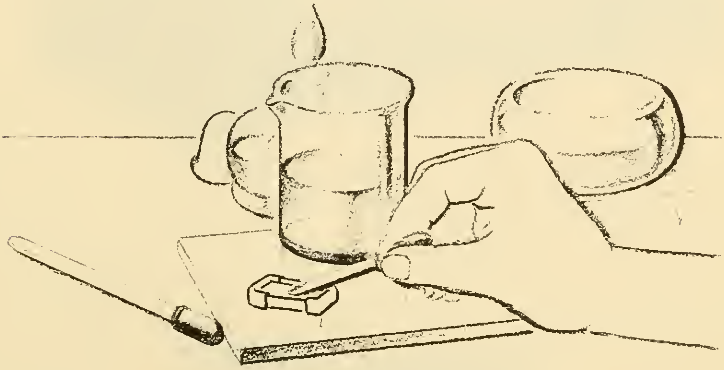


FIG. 30. Remelting the wax around the object with a heated pipette.

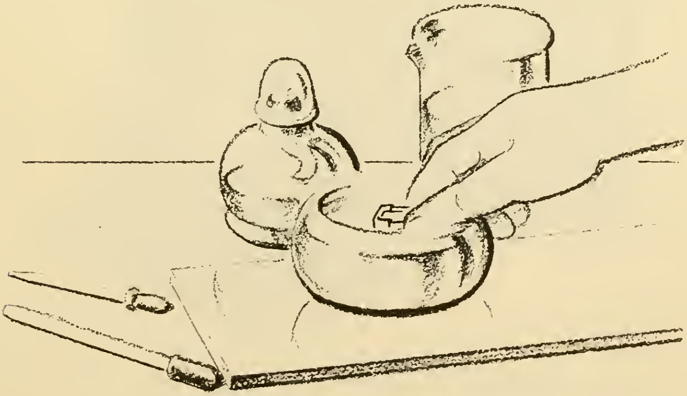


FIG. 31. Cooling the wax block.

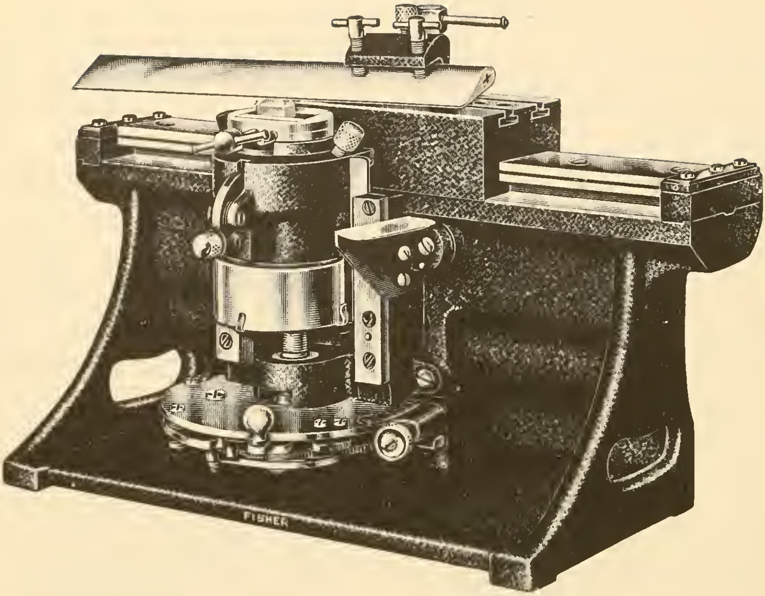


FIG. 32. Sliding microtome.

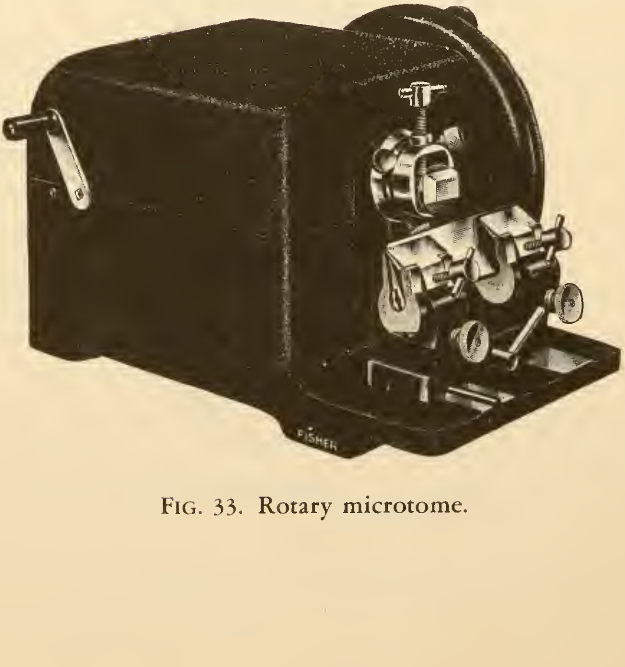


FIG. 33. Rotary microtome.

Choice of a Microtome. Microtomes may be broadly divided into two classes. In the first of these the block remains stationary, while the knife is moved past it. In the second group are those in which the block moves past a stationary knife. The first class (Fig. 32) is commonly known as a Schantz microtome (after the original German model). They are made by several manufacturers, and are rarely used for the preparation of serial sections. They have the advantage that relatively large blocks may be cut but they have the disadvantage that no ribbon can be obtained broader than the width of the knife.

A Spencer or rotary microtome is shown in Fig. 33. In this type of microtome, the rotation of the large wheel causes the block holder to move vertically up and down in most instances through a distance of about three inches. The portion which slides up and down has, at the end opposite the block, a rectangular plate of hardened steel inclined at an angle of about 45 degrees. Under the pressure of a powerful spring, this plate bears against a hardened steel knob which is itself connected to a micrometer screw. As the handle is rotated, a pawl works against a ratchet to move the micrometer screw and thus the knob connected with it through a given distance for each rotation. As the knob moves forward, it bears on the diagonal plate which moves the block the required distance forward at each revolution. This mechanism is very costly to make and is liable to have a large number of minor defects which are not always apparent until one has started section cutting. One of the most important things to watch is that the knob which controls the section thickness is so moved that an exact number of microns is indicated. If, for example, the knob is moved so that the indicator line lies between 9 and 10 microns, the pawl will not engage the ratchet perfectly but will chip off a small portion of brass at each revolution. It requires only a few weeks operation under these careless conditions to destroy the ratchet wheel, which will have to be replaced at the factory. No inexperienced student should ever be trusted with one of these machines until the mechanism of it has been explained and clearly demonstrated to him.

Knives and Knife Sharpening. The most important single factor in the production of good sections is the knife used in cutting. It does not matter how much care has been taken in the preparation of the block or how complex a microtome is used; if the knife-edge is not perfect, there is no chance of securing a perfect section. Ordinary razors are not satisfactory for the production of fine sections. It is necessary to secure a microtome knife, preferably from the manufacturer of the microtome. Another type of microtome knife employs the edge of a safety-razor blade in a special holder; this does not, in the author's opinion, give as good results as a solid blade.

Three types of solid blades are available. First are those which are "square ground"; that is, in which the main portion of the knife is a straight wedge. Second are those which are "hollow ground"; that is, in which both sides of the knife have been ground away to a concave surface, resulting in a relatively long region of thin metal toward the edge. Third are knives which are "half ground"; that is, knives in which one side is square or flat ground and the other side hollow ground. This last type of knife, which the author prefers, is a compromise. There is no doubt that a square-ground knife is sturdier than a hollow-ground knife, a point of some importance when cutting large areas of relatively hard tissues, but there is also no doubt that a hollow-ground knife can be brought more readily to a fine edge. Where a half-ground knife is employed, the flat side should always be toward the block. Microtome knives must be sharpened frequently, but it is necessary, before discussing how to do this, to give a clear understanding of the nature of the cutting edge itself.

If a wedge of hardened steel were to be ground continuously to a fine edge, as in Fig. 34A, it would be utterly worthless for cutting. After only a few strokes, the fine feather edge, which would be produced by this type of grinding, would break down into a series of jagged saw teeth. A microtome knife or, for that matter, any other cutting tool must have ground on its cutting edge a facet of a relatively obtuse angle, whether it is a square-ground knife, as in Fig. 34B, or a hollow-ground knife, as in Fig. 34C. The process of applying this cutting facet to the tip is known as "setting." It is an exceedingly difficult operation to conduct but one which must be learned by every user of a microtome knife. The actual "grinding" of the blade itself to the correct angle or to the correct degree of hollowness cannot be done in a laboratory; the knife must be returned to the manufacturer or to some scientific supply house equipped with the special machinery necessary. The cutting facet, however, must be set at least once a day if the blade is in continuous use. The nature and purpose of this cutting facet is best explained by reference to the mechanism of cutting shown in Fig. 34D. Notice first that the knife blade itself must be inclined at such an angle to the block that the cutting facet is not quite parallel to the face of the block. There must be left "a clearance angle" to prevent the knife scraping the surface every time that it removes a section. In cutting wax, this clearance angle should be as small as possible, and it is for this reason that the blade holder of a microtome is furnished with a device for setting the knife angle. The knife angle should not be set with reference to any theoretical consideration but with regard only to securing this small clearance angle. The only way to judge whether or not a satisfactory clearance angle has been obtained is to observe the sections as they come from the knife.

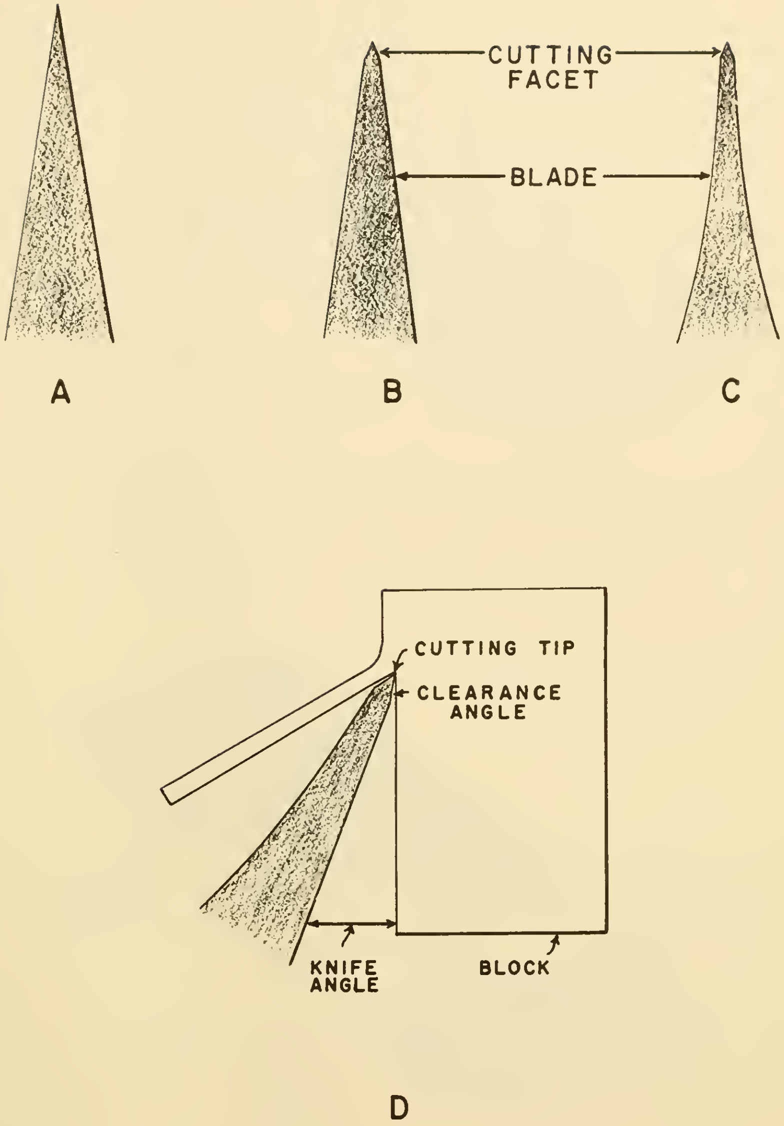

CUTTING FACET
BLADE
A
B
C
CUTTING TIP
CLEARANCE ANGLE
KNIFE ANGLE
BLOCK
D

FIG. 34. Types of cutting edge and cutting action.
(A) Simple wedge without cutting facet.
(B) Flat-ground razor showing cutting facet.
(C) Hollow-ground razor.
(D) Cutting action of knife on wax block.

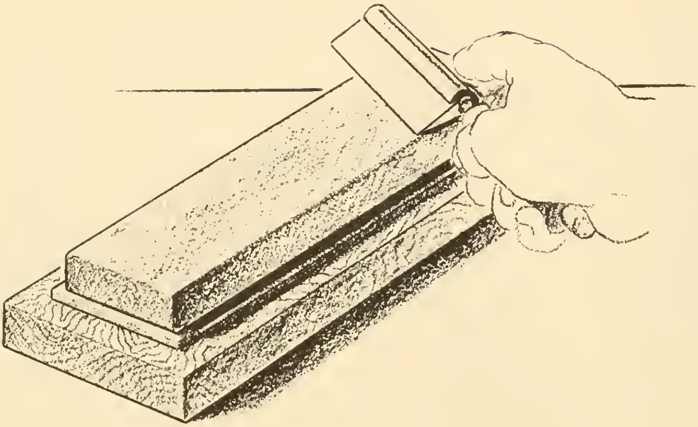


FIG. 35. Setting the cutting facet.

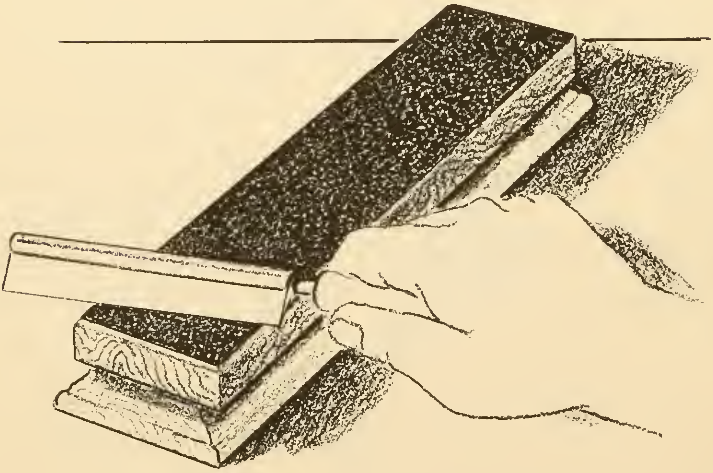


FIG. 36. Stropping a microtome knife.

If the clearance angle is too large, so that the section is not being cut from the block but is being scraped from it, the section will have a wrinkled appearance and will usually roll up into a small cylinder. If the clearance angle is too small, so that the lower angle of the facet is scraping the block after the tip is passed, the whole ribbon of sections will be picked up on the top of the block, which will itself crack off when the knife point reaches it. It is obvious that the knife angle will be changed as the angle of the cutting facet is changed, so that it is desirable to maintain the cutting facet of as uniform an angle as possible. This angle is set on the knife in the manner shown in Fig. 35. Notice that the knife has been furnished with a handle and that a small split cylinder of steel has been slipped over the back of the blade. This split cylinder rests flat on the stone as does the edge of the blade, so that when the knife is pushed forward (Fig. 35 shows it at the beginning of the stroke) the cutting facet is produced as the angle between the cutting edge lying on the stone and the enlarged temporary back which has been placed on the knife. Since a much blunter cutting facet is required for hard materials than for soft, it is strongly recommended that either two knives or at least two sharpening backs be secured. It does not matter what kind of stone is used for sharpening, provided that it is of the finest obtainable grit, that it is dead flat, and that under no circumstances whatever is it used for any purpose except the sharpening of microtome knives. It does not matter whether it is a "water stone," lubricated with glycerin, like the "water-of-Ayr" stones generally used in Europe or an "oil stone," lubricated with mineral oil, like the "Pike" stones so commonly employed in the United States. However, it does matter that it should be flooded with lubricant before starting, and that the knife should be drawn with a light pressure (notice that the finger is *behind* and *not* on top of the knife in the illustration) the entire length of the stone at each operation. If only the central portion of the stone is used, it soon becomes hollowed out, and it is thus impossible to maintain a uniform angle. About three strokes on each side of the knife are quite enough to produce a perfectly sharp cutting facet, and further strokes will have no effect other than to diminish the length of life of the knife.

This direction for the use of three strokes in setting applies, of course, only to a knife which has been treated reasonably and not to that which through carelessness has acquired a nick in its edge. Where the nick is large, it is almost impossible to remove it in setting because the continual setting merely grinds away the edge of the knife and ultimately alters the thickness of the blade itself. If the knife-edge is nicked to a deeper extent than about a quarter of a millimeter, the only thing to do is either to return the knife to the manufacturer to be reground or to avoid that portion of the blade containing the nick when cutting sections. It must be emphasized that the only purpose of setting is to produce a cutting facet and that grinding, which cannot be done in the ordinary laboratory, is required for the removal of knife imperfections. The next question is that of "stropping" the blade of the knife by pulling it backward across a leather surface in the manner shown in Fig. 36. If the knife has been set properly, stropping, the only purpose of which is to polish the facet, is quite unnecessary. The nature of the leather surface which is used for stropping makes it obviously impossible to pull the knife blade forward, and there is a grave risk in pulling it backward that the facet, instead of becoming polished on its flat surfaces, will become rounded on its edges and thus undo the work of setting. Certainly no beginner should be permitted to use a strop until he has demonstrated his ability to set a knife-edge to the point where it will cut an excellent section without stropping. It is also strongly recommended to the beginner that he examine the edge of a knife under the low power of a microscope before setting, after setting, and after stropping.

Mounting the Block. After the knife has been sharpened and the microtome has been selected, the block is trimmed to the correct shape and attached to the object holder of the microtome. The rough block of wax containing the object first must be removed from whatever was used to cast it in or, if a paper box was used, the box cut away roughly with a knife. The block should now be held against a light so that the outlines of the contained object can be seen clearly. The block is trimmed until the object lies in the center of a perfect rectangle with the major axis of the object exactly parallel to the long sides. This is best achieved by finding the major axis at right angles to which the sections are to be cut and by trimming down one side of the block with a sharp safety-razor blade, taking off only a little wax at a time. If one tries to remove a large quantity of wax, there is the danger of cracking the block. After one side is shaved to a flat surface, the other side is shaved parallel to it. The top and bottom surfaces of the block may now be shaved, and it is essential that these should be exactly parallel to each other. A skilled microtomist can cut these edges parallel with a safety-razor blade without very much difficulty, but numerous devices have been described from time to time in the literature to enable one to do this mechanically. It does not matter if these two edges are exactly parallel with the plane of the object; it is essential only that they be parallel with each other. At this stage plenty of wax should be left both in front of and behind the object.

This trimmed block now has to be attached to some holder which can be inserted into the microtome. Since the majority of sections today are cut on a Spencer rotary microtome, the following description is of the use of one of the holders supplied with this machine, although the ingenuity of man has not yet succeeded in devising a worse method of attaching a paraffin block to a microtome. The holder, which is seen in Fig. 37, consists of a disc of metal with a roughened surface attached to a cylindrical shank. First of all, this disc must be covered with a layer of wax, and it is extraordinarily difficult to get wax to adhere to these chromium-plated surfaces. If the worker is not entirely bound by convention, it would be much better for him to secure a series of small rectangular blocks of some hardwood like maple and to soak these for a day or two in molten wax. After they are removed, drained, and cooled, it is the simplest thing in the world to attach a paraffin block to them and to hold them in the jaws of the microtome. Whether the metal holder or the wooden one is used, the technique is essentially the same. A layer of molten wax is built up on the surface and allowed to cool. The block (see Fig. 37) is pressed lightly onto this hardened wax and fused to it with the aid of a piece of heated metal. Some people use old scalpels, but the author prefers the homemade brass tool shown in Fig. 37. Care must be taken to press very lightly with the forefinger and to conduct the whole operation as speedily as possible to avoid softening the wax in which the object is embedded. The metal tool should be heated to a relatively high temperature and applied by just touching it lightly. If the block is very long, it is also desirable to build up small buttresses of wax against each side, being careful not to bring these buttresses so far up the block that they reach the tip of the object to be cut. The metal should now be put aside and allowed to reach room temperature. Many people at this point throw the block and holder into a finger bowl of water, which is all right provided the water is at room temperature. However, there is no more fruitful source of trouble in cutting sections than to have the knife, the block, and the microtome at different temperatures. It is much better to mount the blocks the day before they are to be cut and to leave them on the bench to await treatment. Then final inspection is made of the block to make certain that the upper and lower surfaces of the block are flat, smooth, and parallel. Many people do not make the final cuts on these surfaces until after the block has been mounted in the block holder. The block and the block holder, after insertion in the jaws of the microtome, are seen in Fig. 38. It will be noticed that set screws on the apparatus permit universal motion to be imparted to the block, so that it can be orientated correctly in relation to the knife. It is easy to discover whether or not the edges are parallel by lowering the block until it does not quite touch the edge of the knife, adjusting it until the lower edge is parallel, lowering the block again, and then comparing the relation of the upper edge with the edge of the razor.

Cutting Paraffin Ribbons. The first step in cutting sections on this type of microtome is to make sure that every one of the set screws seen in Fig. 38 is fully tight. The set screws holding the block holder may be tightened in any order, provided that the result leaves the block correctly orientated, but those connected with the knife must be done in the correct order. First the knife is inserted into the holder and fixed firmly but not tightly in place by the two bearings at each end. The tightening of these screws causes the two movable holding arms to hold the knife near its edge. The knife is held in a pair of hemicylinders which may be moved to adjust the knife angle (see Fig. 34D). The knife angle should be set at that which experience has shown to be desirable—no guide other than experience can be used—and the two set screws which lock these inclinable hemicylinders in place then tightened. While the knife is held in place, the two original set screws should be screwed as tightly as the thumb can bear. This leaves the two set screws which come through the inclinable hemicylinders and bear on the back of the knife. These two set screws should be tightened simultaneously and uniformly. The effect of this is to force the knife upward and thus wedge it with extreme firmness in the knife holder.

After all is tight, the handle on the microtome is turned until the block is as far back as possible, and the entire knife moved on its carriage until the edge of the blade is about a quarter of an inch in front of the block. A last minute check is now made to make sure that the divisions of the setting device exactly coincide with the thickness desired. The handle is rotated rapidly until the block starts cutting. The front face will rarely be parallel to the blade of the knife, so that a considerable number of sections will have to be cut until the entire width of the block is coming against the knife. No particular attention need be paid to the quality of this initial ribbon, which may be thrown away.

If all is not going well and the ribbon is not coming off in a perfect condition, refer to Table 1. The remaining operations of preparing and mounting the ribbon are seen far more clearly in illustration than by description. As soon as the ribbon is the width of the knife in length, a dry soft brush, held in the left hand, is slipped under the ribbon, which is then raised in the manner shown in Fig. 38. Care should be taken that a few sections always remain in contact with the blade of the knife because, if the ribbon is lifted till only the edge of the section lies on the edge of the knife, the ribbon will break almost invariably. As the handle is turned, the brush in the left hand is moved away until the ribbon is the length of whatever sheet of paper one has to receive

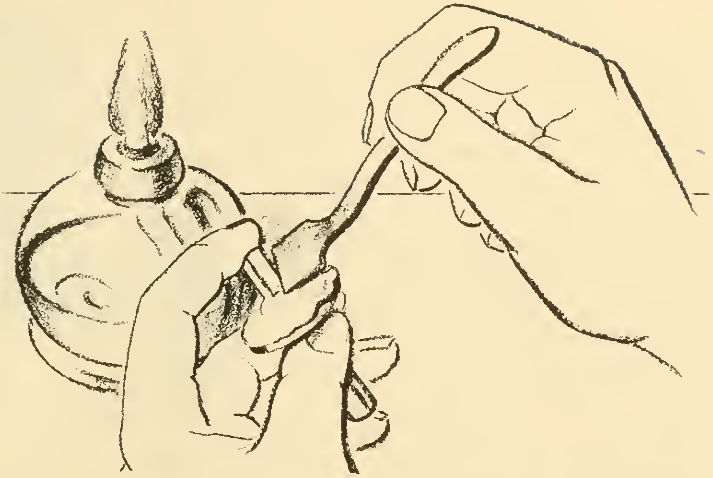


FIG. 37. Mounting the wax block on the block holder.

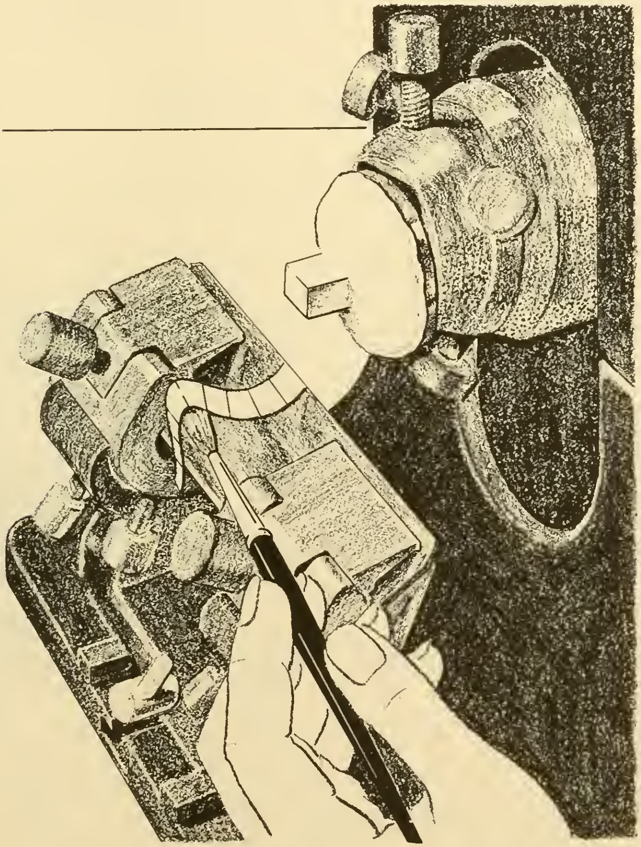


FIG. 38. Starting the paraffin ribbon.

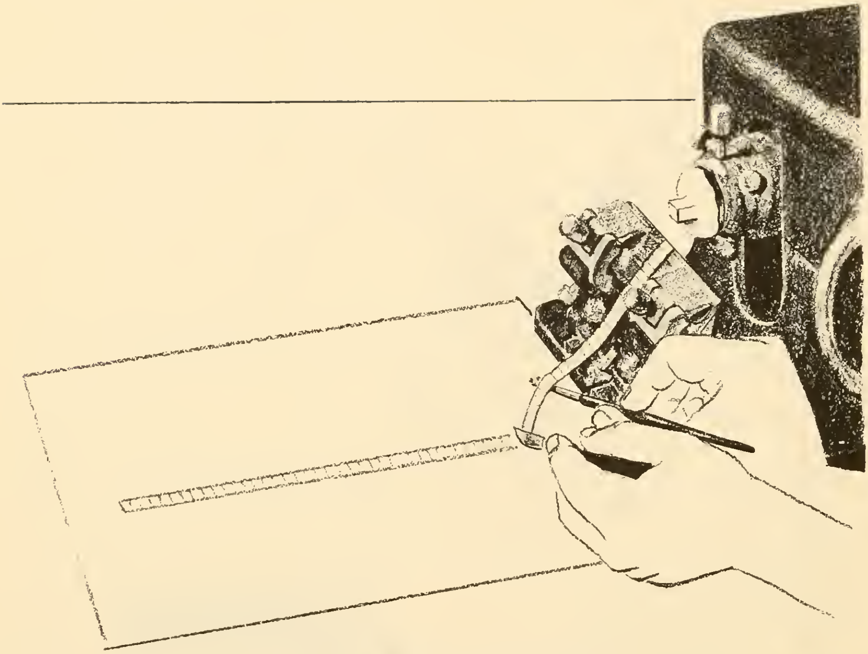


FIG. 39. Laying out the ribbon.

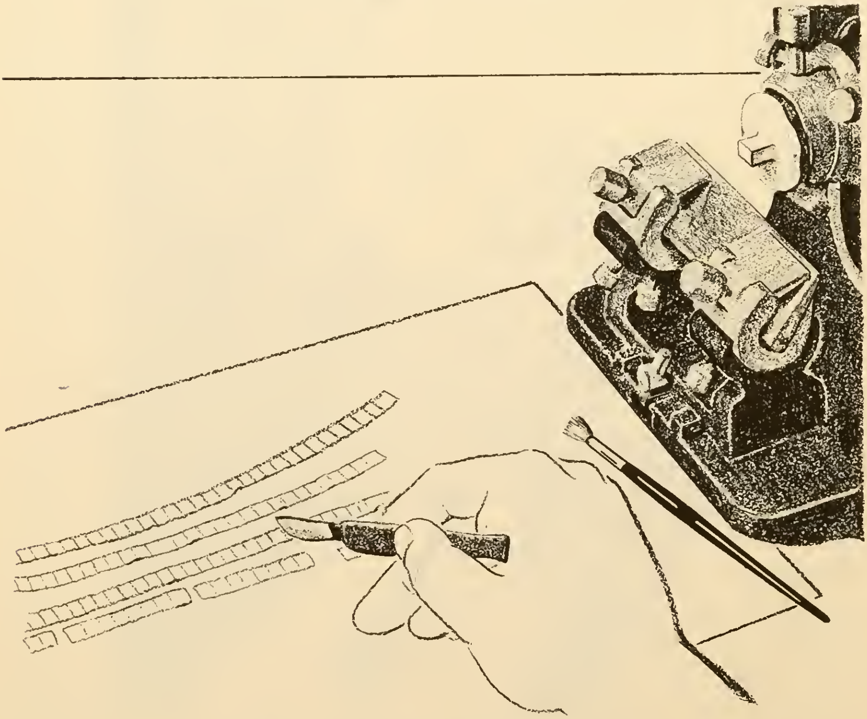


FIG. 40. Cutting the ribbon in lengths.

it on. Legal-size (foolscap) paper is employed quite commonly and is shown in Fig. 39. Notice that the left-hand edge of the ribbon has been laid flat some distance from the edge of the paper and that a loop large enough to avoid strain on the ribbon attached to the knife is retained with the brush, while the ribbon is cut with a rocking motion of an ordinary scalpel or cartilage knife. The larger and colder this scalpel is, the less likelihood there is of the section adhering to it. The purpose of leaving a good margin around the edge of the paper is that it may be desirable to interrupt ribbon cutting for a time and to continue later. In this case the worker should furnish himself with a little glass-topped frame, which is laid over the paper to prevent the sections from being blown about. As the inexperienced worker will soon find out, the least draft of air, particularly the explosive draft occasioned by someone opening the door, is quite sufficient to scatter the ribbons all over the room. These operations of carrying the ribbon out with the left hand, transferring the brush to the right hand, and cutting the ribbon off are continued until the whole of the required portion of the block has been cut and lies on the paper.

The ribbon must be divided into suitable lengths for mounting on a slide (Fig. 40). Although in theory a section should be of the same size as the block from which it came, this practically never occurs in practice, and it is usually safe to allow at least 10 and sometimes 20 per cent for expansion when the sections are finally flattened. The ribbon should never be cut completely until a sample has been flattened on a slide, in order that one may judge the degree of expansion. Though the sections shown in Fig. 41 are mounted on an ordinary 3- by 1-in. slide, it would be more practical for a ribbon as wide as this to use a 3- by 1½-in. or even a 3- by 2-in. slide. The sections should never occupy the whole area of the slide. At least a quarter of an inch should be left at one end for subsequent labeling. When the decision has been made as to how many sections shall be left in each piece of ribbon, the first row of ribbons is cut into the required lengths (Fig. 40). Then the worker must decide what shall be used to cause them to adhere to the slide. It is conventional to use:

MAYER'S ALBUMEN:

Fresh egg white	50 ml.
Glycerin	50 ml.
Sodium salicylate	1 Gm.

The author prefers to dilute the selected adhesive two or three hundredfold with water and to use this diluted adhesive in the next operation of flattening the sections. However, the adhesive can be made full strength and used immediately. If this procedure is desired, shake the ingredients together until they are thoroughly mixed. Filter. Apply a thin smear of this on a clean slide with the tip of the little finger.

It will have been apparent to the worker from the moment he started cutting the sections that they are not absolutely flat. They may be slightly crinkled or slightly distorted and, therefore, must be flattened by being warmed on water heated just below the melting point of the wax. Some people place this water on the slide and then add the sections to it, but the author prefers to lay the ribbons on the slide as shown in Fig. 41. This is not nearly as easy as it looks. The tips of two brushes should be moistened with the tongue just enough to bring the hairs to a point. Then the two moist points are delicately touched (too much pressure will cause the ribbon to adhere to the paper) to each end of the selected piece of section. This piece is lifted as shown in the illustration and placed on the slide. When a sufficient number of pieces of ribbon have been accumulated, the slide is picked up carefully, reversed, and laid on top of the last three fingers of the left hand exactly as shown in Fig. 42. It is quite fatal to grasp the slide by the sides; if this is done when the water is flooded on from the pipette, the meniscus coming to the edge of the slides will break against the fingers, causing the sections to adhere to the fingers permanently. The manner shown is quite safe. The water containing the adhesive (if none has been applied to the slide) is flooded on from a pipette as illustrated. Plenty of fluid should be applied and should be raised in quite a sharp meniscus from the edge of the slide.

The sections must now be flattened. This is much better done rapidly with a flame than slowly on a hot plate. Fig. 43 shows the slide held over a small alcohol lamp, but a micro-bunsen can be employed equally well. The slide should be exposed to heat for a moment, withdrawn to give time for the heat to pass from the glass to the fluid, rewarmed, and so on until the sections are observed to be flat. The utmost care must be taken at this point, for, if the paraffin is permitted to melt, it will be difficult later, if not impossible, to cause the sections to remain attached to the glass. As soon as the sections are flattened, the slide is gently tilted backward toward the hand to run off the excess water against the thumb, leaving the sections stranded in place. The slide is usually placed on a thermostatically controlled hot plate (seen in Fig. 52) and permitted to dry. Most people leave their slides overnight, but frequently an hour would be sufficient. Dryness can be gauged without the least trouble by the fact that a moistened slide shows the wax to be more or less opalescent, while on a properly dried slide it is almost glass-clear.

The method just described is susceptible of several variations, which may

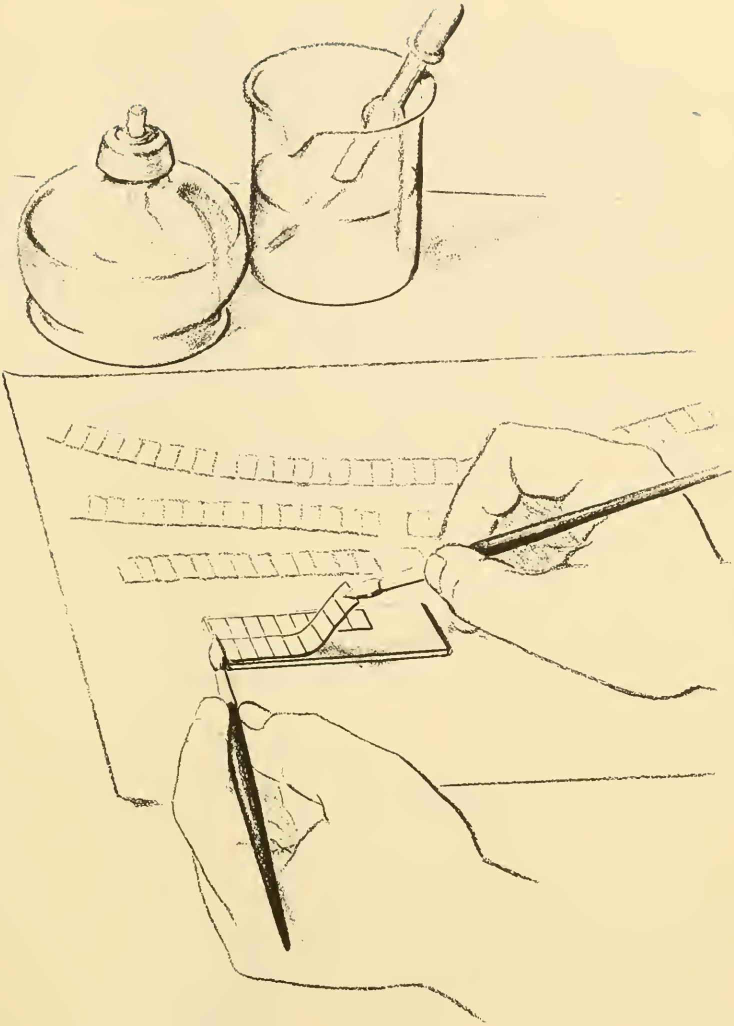


FIG. 41. Mounting the dry ribbon.

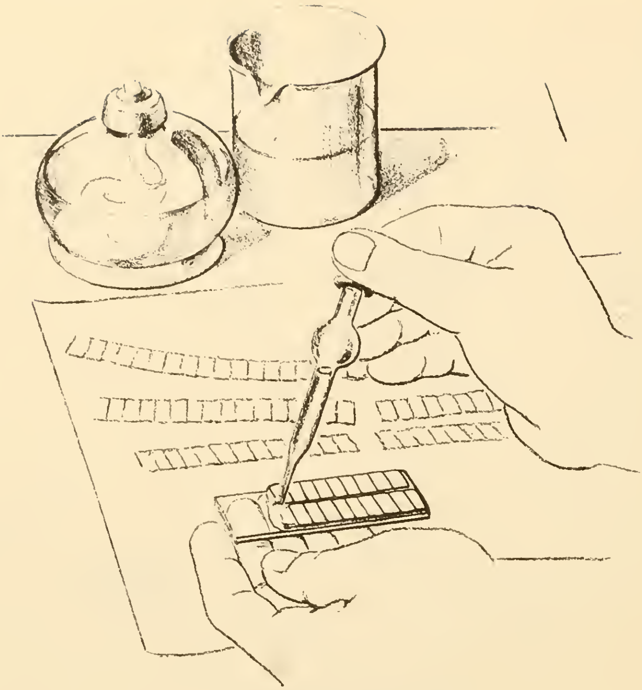


FIG. 42. Flooding the ribbons.

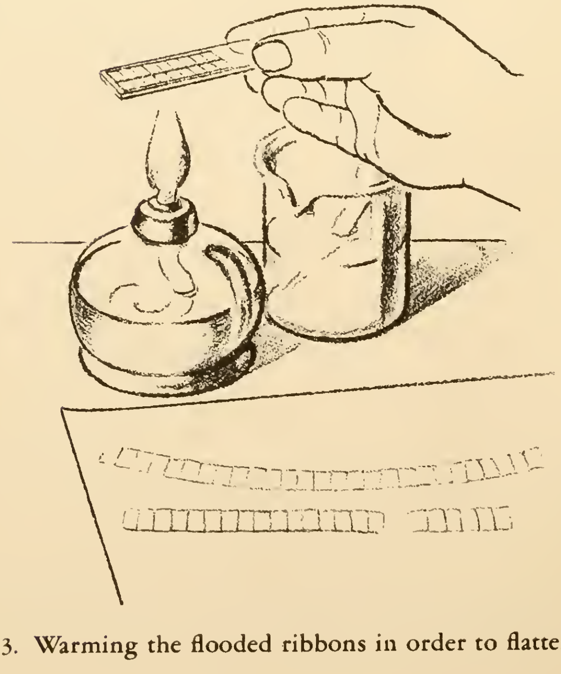


FIG. 43. Warming the flooded ribbons in order to flatten them.

be noted briefly. Some people neither drain the water from the slide nor heat the slide over the lamp but merely place it, as soon as the water has been added to it, on the thermostatically controlled hot plate. This permits the sections to dry and to flatten at the same time. The objection to this procedure is that contained air in the water used for flattening almost invariably comes out in the form of bubbles, which accumulate under the sections, either causing them to fall off or at least making it very difficult to observe properly when mounted. There is also the risk in this procedure that the water will not stop at the edge of the slide but will flood off it unexpectedly, carrying the sections with it onto the surface of the hot plate.

Another procedure, which is not recommended for the inexperienced, is to blot the sections before putting them on the hot plate. If one takes the slide after the water has been drained from it and lays on its surface a *water-saturated* piece of *coarse* filter paper, one can then press hard on the paper with a rubber roller, squeezing much of the water out of both the paper and the sections. This assures that the sections are perfectly flattened in contact with the slide, but requires a strong nerve to try for the first time because of the fear that the sections will stick to the paper. This has not happened in a good many thousand slides which the author has made by this means, and slides so prepared are always free of air bubbles.

Before proceeding to a discussion of the next steps to be taken, it may be well to review the innumerable things that may happen to prevent the production of a perfect ribbon. The appearance, cause, and cure of the more common defects are shown in Table 1. These are by no means the only defects or the only cures which may be applied and every user of the microtome should have in his hands O. W. Richards' "The Effective Use and Proper Care of the Microtome," 1949, which lists many suggestions beyond those here given.

Staining and Mounting Sections. Assuming that all the difficulties mentioned in the last section have been overcome and that one now has a series of slides bearing dried consecutive ribbons, the next thing to be done is to remove the paraffin in order that they may be stained. It is conventional though probably not necessary to warm each slide over a flame (holding it as shown in Fig. 43) until the paraffin is melted thoroughly. Then the slide is dropped, as shown in Fig. 51, into a jar containing xylene, benzene, or some other suitable paraffin solvent.

It is necessary through the subsequent proceedings to be able to recognize instantly that side of the slide on which the section lies. This is not nearly as easy as it sounds, and a lot of good slides have been lost by having the sections rubbed off them. The simplest thing to do is to incline the slide at such an angle to the light that, if the section is on top, a reflection of the section is seen on the lower side of the slide. A diamond scratch placed in the corner is of little use because it becomes invisible when the slide is in xylene. The greatest care should be taken to remove all the wax from the slide before proceeding further, and it is usually a wise precaution to have two successive jars of xylene, passing the second jar to the position of the first and replacing it with fresh xylene after about 10 or 12 slides have passed through. It must be remembered that paraffin is completely insoluble in the alcohol which is used to remove the xylene, so that it is of no use to soak a slide in a solution of wax in xylene and imagine that it will be sufficiently free from wax for subsequent staining. Some people use three jars, the first two containing xylene and the third having a mixture of equal parts of absolute alcohol and xylene, to make sure that all the wax is removed. If even a small trace of wax remains, it will prevent the penetration of stains. Assuming that one is proceeding along the classic xylene-alcohol series, one then passes the slide from either the fresh xylene or the xylene-absolute alcohol mixture to a coplin jar of absolute alcohol. It is unfortunate that as yet nobody seems to have placed on the market a coplin jar or slide-staining dish which has a lid that fits tightly, since absolute alcohol, which is very hygroscopic, is rarely of much use after it has been left on an open bench for a day or two. It does not matter much if xylene is carried over into the absolute alcohol, but as soon as the first trace of a white flocculent precipitate appears in the alcohol—indicating that some wax is being carried over—it must be replaced by fresh alcohol.

The author never bothers to use a series of graded alcohols between absolute alcohol and water. These graded series are necessary, of course, when one is dealing with the dehydration of whole objects which may be distorted, but the author has never been able to find the slightest difference in a thin section between one which has been passed from absolute alcohol to water and one which has been graded laboriously down through 90 per cent, 80 per cent, etc., the length of the series varying upon the wishes of the individual. As soon as the slide has been in water long enough to remove the alcohol, it should be withdrawn and examined carefully to make sure that it has been sufficiently dewaxed. If the water flows freely over the whole surface, including the sections, it is safe to proceed to staining by whatever manner is desired. If, however, the sections appear to repel the water or there is even a meniscus formed around the edge of a section, it is an indication that the wax has not been removed and that the slide must again be dehydrated in absolute alcohol, passed back into a xylene-alcohol mixture, and thence again into pure xylene.

In the specific examples given in the second part of this book, descriptions

Table 1

Defects Appearing While Sections Are Being Cut

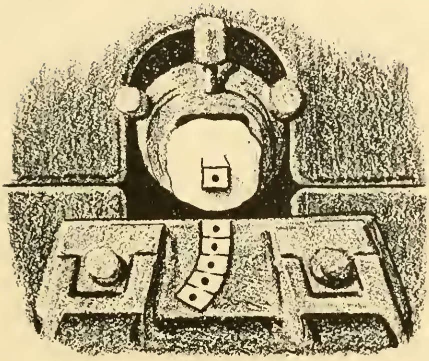


Fig. 44. Ribbon curved.

Possible Causes

1. Edges of block not parallel
2. Knife not uniformly sharp, causing more compression on one side of block than other
3. One side of block warmer than other

Remedies

1. Trim block
2. Try another portion of knife-edge or resharpen knife
3. Allow block to cool. Check possible causes of heating or cooling, such as lamps or drafts

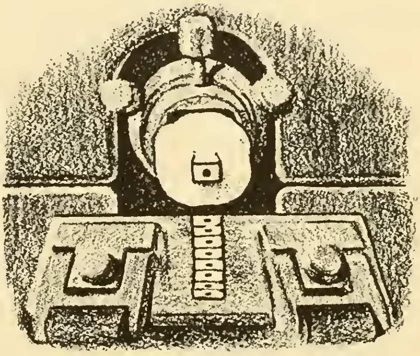


Fig. 45. Sections compressed.

Possible Causes

1. Knife blunt
2. Wax too soft at room temperature for sections of thickness required
3. Wax warmer than room temperature

Remedies

1. Try another portion of knife-edge or resharpen knife. Compression often occurs through a rounded cutting facet (see Fig. 34) produced by overstropping
2. Reëmbed in suitable wax or cut thicker sections. Cooling block is rarely successful
3. Cool block to room temperature

Table 1 (continued)

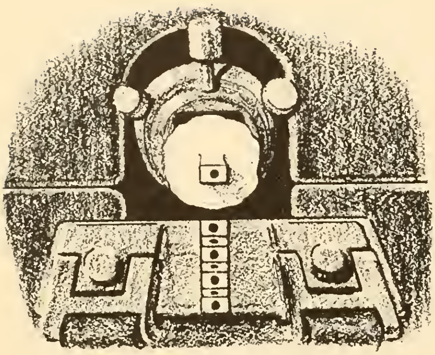


FIG. 46. Sections alternately thick and thin, usually with compression of thin sections.

Possible Causes

1. Block or wax holding block to holder still warm from mounting
2. Block or wax holding block to holder cracked or loose
3. Knife loose
4. Knife cracked
5. Microtome faulty

Remedies

1. Cool block and holder to room temperature
2. Check all holding screws. Remove block from holder and holder from microtome. Melt wax off holder and make sure holder is dry. Recoat holder and remount block. Cool to room temperature
3. Release all holding screws and check for dirt, grit, or soft wax. Check knife carriage for wax chips on bearing
4. Throw knife away
5. Return microtome to maker for overhauling

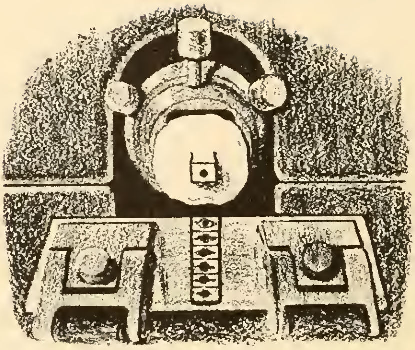


FIG. 47. Sections bulge in middle.

Possible Causes

1. Wax cool in center, warm on outside
2. Only sharp portion of knife is that which cuts center of block
3. Object impregnated with hard wax and embedded in soft, or some clearing agent remains in object

Remedies

1. Allow block to adjust to room temperature. This is the frequent result of cooling blocks in ice water
2. Try another portion of knife-edge or resharpen knife
3. Reëmbed object

Table 1 (continued)

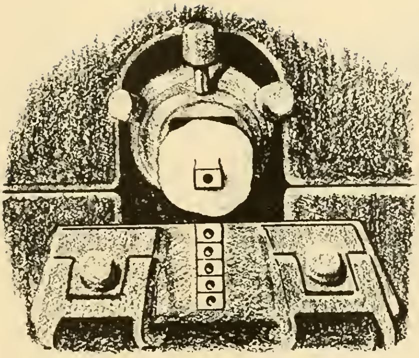


FIG. 48. Object breaks away from wax or is shattered by knife.

Possible Causes

1. If object appears "chalky" and shatters under knife blade, it is not impregnated
2. If object shatters under knife but is not chalky, it is too hard for wax sectioning
3. If object pulls away from wax but does not shatter, the wrong dehydrant, clearing agent, or wax has been used

Remedies

1. Throw block away and start again. If object is irreplaceable, try dissolving wax, redehydrating, reclearing, and reëmbedding
2. Spray section between each cut with celloidin
3. Reembed in suitable medium, preferably a wax-rubber-resin mixture. Avoid xylene in clearing muscular structures

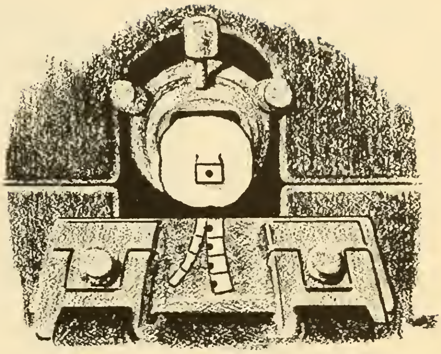


FIG. 49. Ribbon splits.

Possible Causes

1. Nick in blade of knife
2. Grit in object

Remedies

1. Try another portion of knife-edge
2. Examine cut edge of block. If face is grooved to top, grit has probably been pushed out. Try another portion of knife-edge. If grit still in place, dissect out with needles. If much grit, throw block away

Table 1 (continued)

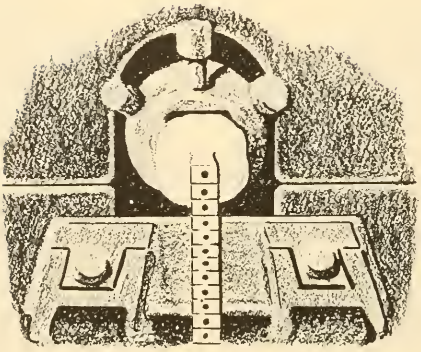


FIG. 50. Block lifts ribbon.

Possible Causes	*Remedies*
1. Ribbon electrified. (Check by testing whether or not ribbon sticks to everything else)	1. Increase room humidity. Ionize air, either with high frequency discharge or bunsen flame a short distance from knife
2. No clearance angle (see Fig. 34)	2. Alter knife angle to give clearance angle
3. Upper edge of block has fragments of wax on it (a common result of 2)	3. Scrape upper surface of block with safety-razor blade
4. Edge of knife (either front or back) has fragments of wax on it	4. Clean knife with xylene

No ribbon forms

Defect	*Possible Causes*
1. Because wax crumbles	1. Wax contaminated with clearing agent
2. Because sections, though individually perfect, do not adhere	2. Very hard, pure paraffin used for embedding
3. Because sections roll into cylinders	3a. Wax too hard at room temperature for sections of thickness required
	b. Knife angle wrong

Remedies

1. Reëmbed. (*Note:* Wax very readily absorbs hydrocarbon vapors)
2. Dip block in soft wax or wax-rubber medium. Trim off sides before cutting
3a. Reëmbed in suitable wax. If the section is cut very slowly, and the edge of the section held flat with a brush, ribbons may sometimes be formed
b. Adjust knife angle

Table 2

Defects Appearing After Sections Have Been Cut

Defect	*Cause*	*Remedy*	*Method of Prevention*
Sections appear wrinkled	1. Blunt knife used for cutting	1. None	1. Sharpen knife and cut new sections
	2. Water used for flattening too hot, so that folds in sections fused into position	2. None	2. Watch temperature of water used for flattening
	3. Sections unable to expand sufficiently a. Because water used for flattening too cold b. Because area of water too small	3. None	3a. Watch temperature of water used for flattening b. Make sure that slide is clean, so that water flows uniformly over it
Sections have bubbles under them	1. Sections insufficiently flattened, so that air is trapped	1. If sections still wet, reflood slide with water and reheat to complete flattening	1. Check flatness of sections before draining slide
	2. Air dissolved in water used for flattening comes out and is trapped under sections in drying	2. If sections still wet, reflood slide with water, work out bubbles, and reheat to complete flattening	2. Use air-free (boiled) water for flattening. Drain slide thoroughly and blot off excess moisture. Squeeze sections to slide (see p. 69)
Sections fall off slide	1. Wax melted in flattening	1. None	1. Watch temperature of water used for flattening
	2. Slide greasy	2. None	2. Use clean slides
	3. Alkaline reagents dissolve albumen adhesive. (Sections start to work loose in course of staining or dehydrating)	3. Transfer slides carefully to absolute alcohol. When dehydrated, dip in 0.5% celloidin in ether-alcohol. Then dip in 50% alcohol to coagulate celloidin. (This is not worth while unless sections absolutely irreplaceable)	3. See Gray's *Microtechnique* for other section adhesives not alkali sensitive
	4. Sections not flattened into perfect contact with slide	4. None	4. Sometimes caused by swelling of sections which causes center to lift. Squeeze sections to slide (see p. 69) and dry as rapidly as possible

Table 2 (continued)

Defect	*Cause*	*Remedy*	*Method of Prevention*
Sections distorted	1. Blunt knife and soft wax	1. None, though prolonged flattening on warm water may help	1. Use suitable knife and embedding medium
	2. Ribbon stretches when picked up on hot day	2. As (1) above	2. Handle ribbons in short lengths or use harder wax
	3. Tissues not properly hardened before embedding	3. None	3. Use more suitable fixative or fix longer. Take extra care in dehydrating, clearing, and embedding
Sections appear opaque or have highly refractive lines outlining cells and tissues	1. Clearing agent evaporated before mountant added	1. None	1. Obvious
	2. Sections insufficiently cleared or cleared in agent not miscible with mountant	2. Soak off cover. Clear properly	2. Check quality and nature of clearing agents and mountants
Sections will not take stain or stain irregularly	1. Wax not perfectly removed before staining	1. Return sections through proper sequence of reagents to xylene. Leave until wax removed. Restain	1. Change first jar of xylene frequently
	2. Section not uniform thickness	2. None	2. See Table 1
	3. Tissue "old" (has been stored for a long time in alcohol or, worse still, fixative)	3. Return sections through proper sequence of reagents to water. Wash overnight. If not effective, consult Gray's *Microtechnique* for "tissue revivers"	3. Store all tissues embedded in paraffin blocks—never liquids
	4. Fixative not suitable before staining technique employed	4. Try mordanting sections in recommended fixative	4. Obvious
	5. Fixative not fully removed	5. See p. 12 for special methods	5. Treat tissues as indicated on p. 12

Table 2 (continued)

Defect	*Cause*	*Remedy*	*Method of Prevention*
Sections contain fine opaque needles	1. Imperfect removal of mercuric fixatives	1. Return sections through proper sequence of reagents to water. Treat 30 min. with Lugol's iodine (see p. 12), rinse, and bleach in 5% sodium thiosulphate. Restain	1. Treat tissues as indicated on p. 12 before embedding
Sections contain black granules	1. Long storage in formaldehyde	1. None	1. Never store tissues in formaldehyde—always in paraffin blocks

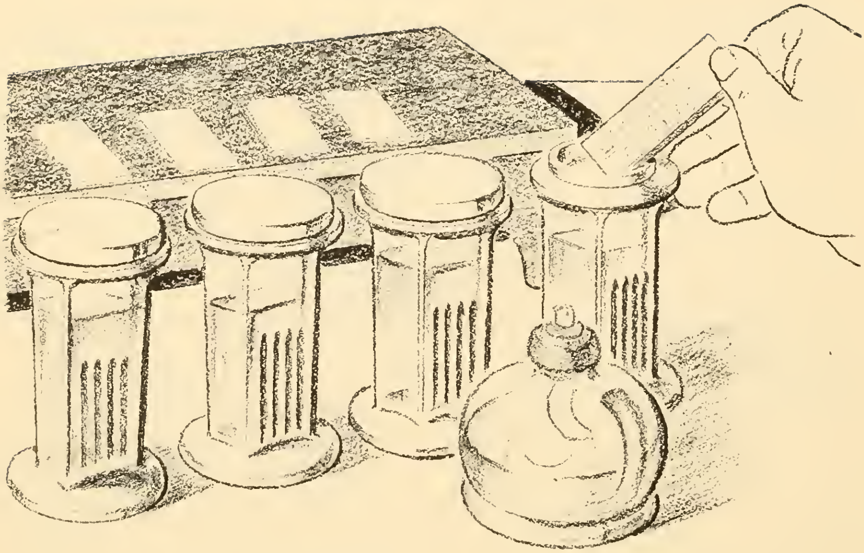


FIG. 51. Starting a slide through the reagent series.

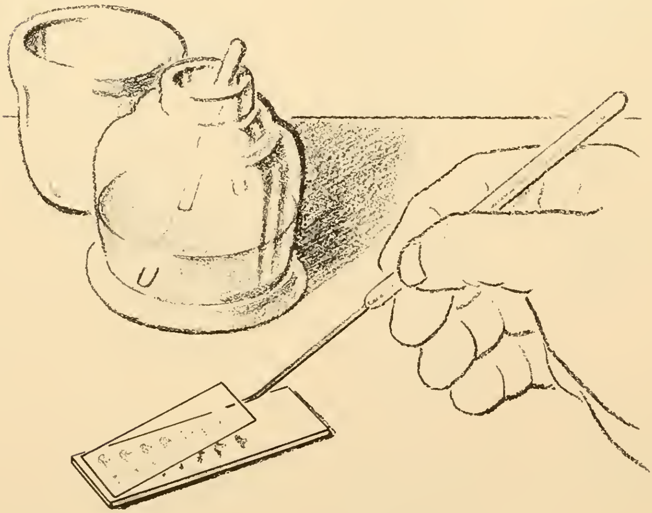


FIG. 52. Placing the coverslip on serial section slide.

are given of individual staining methods. The purpose of this chapter is to discuss only the general principles involved.

It is assumed that the sections will be mounted in a solution of dried balsam in xylene. The slide is removed from the xylene, drained and placed on any convenient flat surface. A drop of the mountant is taken from the bottle and liberally dropped over the surface of the sections. A coverslip of suitable size (Fig. 52) is held at an inclined angle with a bent needle and slowly lowered so as to exclude all air bubbles. Then the edges of the slide are wiped roughly, and the slide is returned to the hot table shown in Fig. 51 to evaporate the solvent used for the resin.

Although this is the conventional method of operation, it is by no means the best. In particular, there is a tendency to have a higher concentration of solvent along the edges of the coverslip than is in the center, and it also takes a surprisingly long space of time for all the solvent to be removed. It is much better, if one can spare the time, to place a relatively thin coat of mounting medium on top of the slide and then to allow the solvent to evaporate from this on the surface of a hot plate. There is no risk that the slide will dry out because the mountant will act as a varnish. It is needless to say that in fine work it is necessary to cover the slide and hot plate with some dustproof cover while this is going on. The next day the slide is examined and, if it appears to be sufficiently varnished, the coverslip is placed on the surface. Then the whole slide is warmed, while maintaining steady pressure, above the softening point of the resin. The slide will be hardened as soon as it is cooled and may be cleaned and put away. This custom of evaporating the solvents from the surface of the slide rather than from the edge of the coverslip is considered old fashioned nowadays, but there is no doubt that it produces a better and more durable slide than does the more usual procedure.

It must not be imagined that, just because all these directions have been followed scrupulously, a perfect slide will result. There are nearly as many things which can go wrong with a section after it has been cut (see Table 1) as there are things that can happen in the course of cutting. Some of the more important of these things are listed in Table 2, but it must be realized that no amount of written instruction can take the place of experience.

Frozen Sections

There are two circumstances under which paraffin sections cannot be used: first, where it is desired to preserve in the tissues some fatty material which would be dissolved out by the reagents used prior to impregnating; and, second, when speed is of primary importance, as in the production of quick sections from tumors for diagnostic purposes. In both these cases, recourse may be had to the method of frozen sections in which material is rapidly frozen until it is of a consistency where it may be cut. Frozen sections should not, however, be employed on any occasion when the normal processes of embedding may be used.

Choice of a Microtome. The type of microtome shown in Fig. 53 is so universally employed that it will be taken as the basis for the present discussion. It is essential in cutting frozen sections that the knife slice, rather than push, through the tissue. This slicing effect is produced by mounting the knife to swing through the object when the handle on top is turned. These microtomes are not as accurate, either as to the thickness of section cut or the repetition of this thickness, as is the big Schantz shown in Fig. 32, but it is to be presumed that no one would cut frozen sections, in any case, if thickness and reproducibility were primary objectives. The method of freezing the object will be discussed after we have dealt with the question of supporting the material in a suitable medium. Even if one can spare only a few moments in excess of the absolute minimum time required to cut without embedding, better results will be obtained if one smothers the object in several layers of:

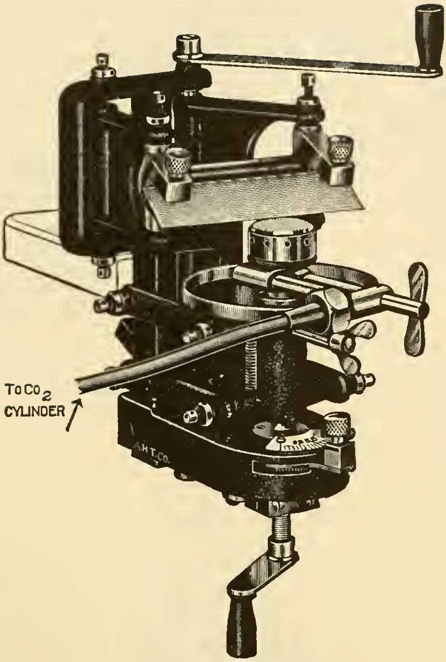
ANDERSON'S MEDIUM:

Simple syrup	50 ml.
80% Alcohol	50 ml.
White dextrin	15 Gm.

Note: The dextrin is boiled to solution in the syrup and cooled; the alcohol is added slowly and with constant stirring. Simple syrup, which may be obtained from any pharmacist, is prepared by dissolving 5 lbs. of sugar in 2 pts. of water.

Choice of Refrigerant. Blocks are nowadays almost invariably frozen with the aid of carbon dioxide which is available very cheaply in large cylinders. The cylinder is connected through a needle valve to the object holder of the microtome, so that one has only to twist the valve to project a jet of super-cooled carbon dioxide against the underside of the object holder.

Process of Cutting. The prime necessity for producing a good section is, of course, the availability of a sharp microtome knife. The nature, care, and sharpening of microtome knives has already been discussed. Assuming that more than the minimum time is available and that the method of Anderson is to be used, the following materials are required: A bottle of Anderson's syrup, which is conveniently kept in a "balsam" bottle, a pipette of the eyedropper type, and a dish of 70 per cent alcohol in which to receive the sections as they are cut. It is to be presumed also that the carbon dioxide cylinder has been attached to the tube leading to the microtome's freezing table and that a brief trial has been run to make sure that the gas is flowing satisfactorily.



To CO_2 CYLINDER
A.H.T.Co.

FIG. 53. Spencer clinical microtome fitted for freezing.

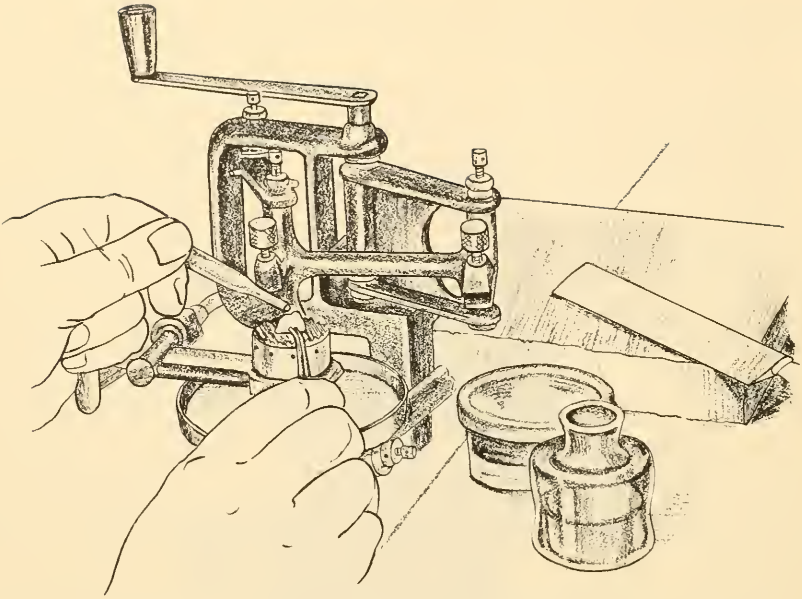


FIG. 54. Applying Anderson's medium to tissue about to be frozen.

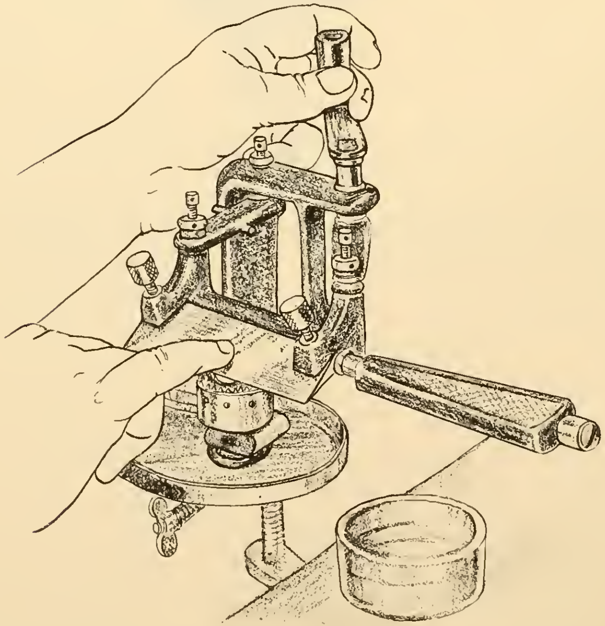


FIG. 55. Removing section from knife.

About half a milliliter of syrup is picked up with the pipette and placed on the freezing table of the microtome. A small jet of carbon dioxide is then turned on. Within a moment or two the gum will be seen to have congealed and the carbon dioxide is turned off. The object to be sectioned is then (Fig. 54) placed on top of this congealed layer of gum and more gum poured over the surface. Care must be taken that a layer of uncongealed gum lies between the object and the congealed gum, or it may loosen. The carbon dioxide is again turned on, and, as soon as the gum covering the object is seen to be congealing, a little more syrup is poured on the surface so that the object is thoroughly covered. It will be seen from the figure referred to that, as a matter of convenience, the knife has not yet been placed in its holder while these preliminary operations are going on. As soon as the knife has been inserted, an experimental cut is taken across the top of the material with the knife, and the block is then shaved down until the specimen is reached. The device which controls the thickness of the section to be cut is then set to whatever thickness has been decided upon. It is strongly recommended that no attempt be made to cut sections much less than 20 microns in thickness by this method, and often sections of 30 microns are sufficiently good for diagnostic purposes. It is then only necessary to continue to pull the handle until the object starts to cut, while observing the nature of the sections. If the sections crumble under the action of the knife, while the gum melts instantly on contact with it, it may be presumed that the block has not been frozen sufficiently hard; the carbon dioxide may again be turned on for a few moments, and another cut taken. It will only take a moment or two to establish the optimum condition under which only slightly curled sections appear on the blade of the knife. As the blade, however, is likely to have become soiled, it is now washed with a drop of warm water to remove the dried gum and then used to cut as many sections as are required. As each section is cut, it must be removed from the blade of the knife to the dish of 70 per cent alcohol shown in the figure. The majority of people working under pressure use their little finger (Fig. 55) for the removal of the section, though a number of very competent technicians prefer to use a brush for this purpose. As soon as the section has been removed, it is transferred to the 70 per cent alcohol where the gum will soon be dissolved.

Cutting without support is much more difficult and should be confined to homogenous blocks of tissue. In this case the tissue, either fresh or fixed in formaldehyde, is trimmed so that a straight edge will be opposed to the edge of the knife. A drop of water is placed on the surface of the freezing table, the gas is turned on, and the specimen is pressed firmly to the water while it is frozen in place. The finger is then removed and the block watched until the line of frost on the side just reaches the top. Cutting should start at once using a rather slow, even stroke. The correct degree of freezing is vital to success but can only be learned by experience.

Mounting and Staining Frozen Sections. Fairly thick frozen sections may be handled with section lifters and passed through any of the stains described in Chapter 3. Sections of fatty tissue are always handled in this manner and should be stained by the method discussed on page 29.

If complex staining techniques are to be applied, however, it is better to fix the section or sections to a slide. One of the best techniques involves two solutions:

GRAVIS' ADHESIVE:

Water	100 ml.
Agar	0.1 Gm.
Camphor	0.1 Gm.

Dissolve the agar in boiling water. Filter and add the camphor to the hot filtrate.

ZIMMERMAN'S LACQUER:

Absolute alcohol	50 ml.
Anhydrous ether	50 ml.
Pyroxylin U.S.P. *or* Parlodion	0.5 Gm.
Gum mastic	0.1 Gm.

The first solution is used exactly as water is used (see p. 68) to flatten a paraffin section. The amount of warming necessary depends on the degree of wrinkling of the section but is usually less than for paraffin ribbons. As soon as the slide has been drained, absolute alcohol is dropped gently on the section from a pipette, left for a few moments, and then drained off. A few moments now suffice to dry the sections, which become opaque. A piece of lens paper is then laid over the slide, and each section is pressed firmly with the finger. The slide is now quickly dipped in absolute alcohol, drained, and dipped in Zimmerman's solution. This last is allowed to evaporate from the surface, leaving behind a coat which will hold the section in place through subsequent staining techniques. Any of the stains discussed in Chapter 3 may be used save those involving differentiation in absolute alcohol which would dissolve the lacquer. The slides are treated exactly as though they carried paraffin sections except that the final transfer should be from 95 per cent alcohol to terpineol to xylene. Even terpineol has a slight solvent action on the slide which should remain in it, therefore, only long enough to remove the alcohol.

9

Cleaning, Labeling, and Storing Slides

There is not much point in going to all the trouble and difficulty of making a good microscope slide unless one is also prepared to finish, label, and store it properly. There is a great deal of difference between a slide which is merely left after the coverslip has been placed on it with a label stuck roughly on the end and one which has been properly finished and properly labeled. This, indeed, is the principal difference between the "professional" slide which one buys from the biological supply house and one which is turned out by the average beginning student.

There are two stages to finishing a slide. The first of these is to clean from the outside all unwanted mountant and to polish the glass. The second is to attach to it a label which is both neat and permanent. It is easy to remove the unwanted gum mountant from wholemounts if one is using either the medium of Farrants or the medium of Berlese, for it is necessary only to wipe very gently with a damp cloth until the surplus has been removed. If far too much of the medium has been used, so that there is a large exudate around the edge of the coverslip, it is usually better to work in two stages; that is, to remove about half of this exudate one day and the remainder the next day. The reason for this is that these gums harden only on the outside and, if the whole of the surplus is washed off at one time, there is a grave risk of displacing the coverslip. A somewhat different procedure is employed when one is cleaning a slide which has been mounted in the medium of Gray and Wess. This material dries to a tough pellicle which is not water soluble but is easily removed with a knife. The sharp point of a scalpel is run either around or along the edge of the coverslip, and then the surplus hardened pellicle is picked off as a single sheet. The cut must extend all the way through to the glass or the coverslip, with the object attached to it, may come off. However, this is not a permanent catastrophe since one can easily remount the coverslip with its adherent object in the same medium.

In either case, after the surplus medium has been removed, the slide is left for at least two days to harden, dipped very briefly in a finger bowl of a cold soap solution, and then dried and polished. The slide must not be left in the soap long enough to loosen the coverslip, or the coverslip is likely to be removed when polishing the slide.

Cleaning wholemounts made in resinous media is a rather different procedure. It is necessary to wait until the resin has completely hardened, hastened where necessary by exposure on a hot plate. Then as much of the surplus as can be removed is scraped off with a dull knife. A small lump of absorbent cotton saturated in 96 per cent alcohol is used to rub off the remainder of the resin. It is not safe to endeavor to clean a balsam slide with benzene or xylene, for the coverslip will be loosened inevitably by this method. As soon as the surplus resin has been dissolved away, the slide is dipped immediately into a warm soap solution and then polished. This leaves a very brilliant finish and an entirely clean slide.

An additional reason why slides should always be washed in soap and water after they are mounted is because there is no known method by which a gummed paper label may be made to adhere to greasy glass. More slides are rendered useless through the loss of their label than from any other defect. Almost everyone today buys pregummed paper labels, so that it is scarcely worth while in this text to give formulae for label adhesives. There is, however, a very definite technique by which the label may be made to stick most readily. This is to lick the *upper* surface of the label, moisten thoroughly the lower surface, and then, when both sides are fully expanded, to press it firmly on the glass. Labels attached in this manner have remained for more than 20 years in the author's collection without becoming detached. One may either then wait until the label is dry before printing neatly on it with waterproof India ink the name of the specimen or, if one is dealing with a large series of slides, one may write the label first in waterproof India ink and, having left it at least 24 hours to dry, attach it by the method indicated.

Even if the label is attached by the method indicated, it is always best, in the case of valuable slides, to write a brief label with a writing diamond on the glass underneath the label.

There is only one absolutely permanent method of labeling a microscope slide, and that is to use a slide of which about an inch at the end has been ground to a rough surface. After the slide is finished and cleaned, the label is written on this ground surface either with a soft pencil or with waterproof India ink. Then a drop of balsam is placed on the label before attaching a coverslip over the the surface of it. The objections to this method are that it is very expensive—the slides cost four or five times as much as ordinary slides—and also that there is a grave risk that the coverslip will be broken by someone placing the clip of the microscope stage on top of it.

The proper storage of microscope slides is just as necessary as proper labeling. Slides should always be stored in the dark and in as cool a place as possible because none of the stains used in biology are absolutely permanent. Slides may be stored either vertically on edge in grooves in a tray of a cabinet of the type shown in Fig. 56 or lying flat on the bottom of a tray as in the

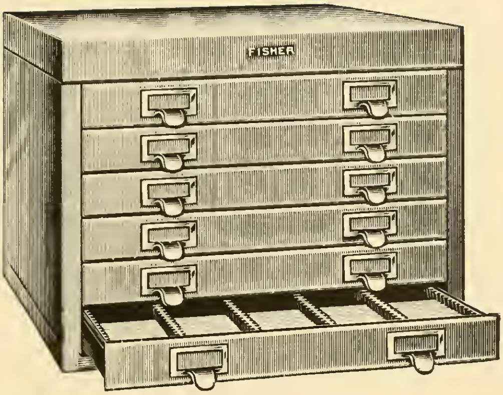


FIG. 56. Slide storage cabinets with vertical grooves.

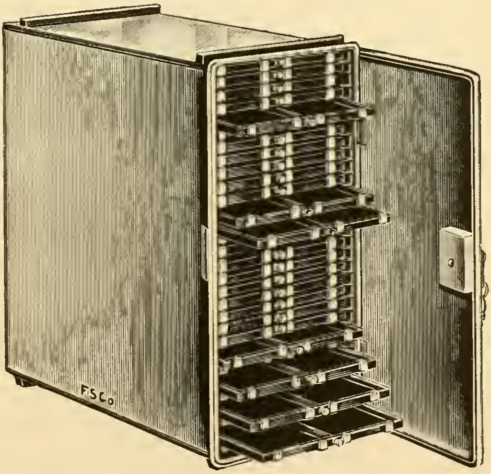


FIG. 57. Slide storage cabinet for flat storage.

type of cabinet shown in Fig. 57. There are arguments for and against both methods of storage. Slides which are stored vertically occupy much less space than do those which are stored flat. The former method is not very suitable for wholemounts for the reason that the interior of the slide never dries and, after a slide has been stored for some years in a vertical position, the object will be found to have dropped down to the edge of the coverslip and become embedded in the hardened balsam, from which it is very difficult to detach it later. There is, however, no objection to the storage of sections on edge, and many thousands of slides stored by this method can be kept in the space which is occupied by only a few hundred when they are stored flat.

Another method of storing slides, which is particularly useful in the case of serial sections involving many dozens of slides in each series, is to take two 3- by 5-in. index cards and to cut about an inch from the long edge of one of them. They are stapled together, making a series of pockets into each of which a slide may be inserted. The full data connected with the slide may be written on the card, which may be stored in any of the ordinary card-filing cabinets that are available.

There are many types of small boxes made which have grooves to hold the slides. It is to be remembered with these that the slide must be kept in a horizontal position if it is a wholemount.

There is a considerable case to be made out for "ringing" the edges of coverslips with a colored varnish not only to improve the appearance of the slide but also to increase its permanence. This procedure is described in full in Gray's *Microtechnique,* and space does not permit it to be given in the present text.

Part 2

Specific Examples of Slide-Making

Example 1

Preparation of a Wholemount of a Mite by the Method of Berlese

The use of the name, Berlese, in the heading of this example is less an injunction to employ the mounting medium of that writer than it is a tribute to the method of collecting small arthropods, which he introduced. This method uses the "Berlese funnel." This device is a double-walled funnel, between the walls of which warm water may be placed and maintained at any desired temperature by applying a small flame to a projecting side arm. The temperature is not critical, so that no thermostatic mechanism is provided, but a thermometer may be inserted and used to read the temperature at intervals. A circle of wire gauze with a mesh of about a sixteenth of an inch is placed at the bottom of the inner glass funnel and the material that is to be searched for mites is placed loosely on this gauze. The lower end of the glass funnel is attached with modeling clay to a tube containing whatever medium is being used for the collection of the specimens. If the specimens are to be stored rather than mounted at once, 96 per cent alcohol may be placed in the tube, and it is unnecessary to seal it at the base of the funnel. If, however, the specimens are to be mounted at once in Berlese's medium, in which much better mounts can be prepared from living than from preserved material, the tube must contain water and be sealed to the funnel in order to prevent the more active forms from working their way out of the tube.

After the moss has been placed in position, a small lamp of not more than 15 watts is mounted in any kind of a reflector some distance above the material. The animals in the material, therefore, find themselves surrounded by heat at the sides and plagued with light from above. As all of these animals are violently photophobic, they tend to move automatically toward the lowest point of the moss, from which they drop down the funnel into the tube. By this means it is possible in 10 or 15 minutes to collect the whole fauna from a large handful of any organic material which would, by any other means, take several hours to search. The use of the funnel is not confined to moss but may be applied to hay, straw, shredded bark, or indeed any other material from which small arthropods are customarily collected. The only difficulty in using this equipment is in preventing the heat from becoming too great. Some people use so large a lamp above and so high a temperature around the edges, that many small arthropods are killed before they have time to fall into the trap which has been laid for them. The author has found that the water between the walls of the funnel for most uses should be at a temperature of

30 to 40° C., while the lamp above should under no circumstances raise the surface temperature of the material above 60° C. These temperatures are for a moderately dry moss sample and may be exceeded greatly when one is dealing with dry material, such as straw. Wet moss of the sphagnum type, however, requires lower temperatures if it is to be examined successfully.

If permanent mounts are to be made for record purposes of all the small invertebrates which may be found in the moss sample, it is necessary to make adequate preparations to receive the animals while the moss is being treated. Two kinds of gum mountants are desirable: (1) A high refractive-index medium like Berlese' for the very heavy-walled forms, such as the Oribatid mites and the pseudoscorpions and (2) a low refractive-index mountant like Gray and Wess', for the thinner-walled forms, such as the Tyroglyphid and Gamasid mites. This last medium is also suitable for Thysanura and for Collembola. Thick-walled beetles and fleas, if they are to be made into microscope slides, had better be treated with alkali and should be accumulated for this purpose in a tube of 96 per cent alcohol. This process is described in detail in Gray's *Microtechnique.* It consists usually of soaking the specimens after rehydration in 10 per cent potassium hydroxide. Excessive swelling may be controlled by enlarging the anus with a needle and by putting holes in unimportant parts of the head and thorax.

If one is dealing with a sphagnum moss, it is also possible that a number of crustaceans, particularly Cladocera and Ostracoda, are likely to be found. These are better mounted in glycerin jelly in the manner also described in Gray's *Microtechnique* and should be transferred, as soon as they are found, to 30 per cent alcohol where they will die with their appendages extended. They should not be permitted to remain in this weak alcohol for longer than is necessary to kill them. Then they should be transferred to 96 per cent alcohol. A large number of nematode worms are likely to turn up; these cannot be mounted by any of the methods described in this book, and again reference should be made to the author's larger work. A tube of some fixative, a supply of clean 3- by 1-in. glass slides, and a number of coverslips should be provided to receive any small annelids which may be found in the moss, and which must be fixed, stained, and mounted at once.

When all is ready and observation shows that no more forms are falling through the Berlese funnel, the collecting tube beneath it is inspected to see roughly what one has gathered. If there are a great number of Gamasid mites or active insects, it is necessary to open gently a portion of the tube by pushing away the modeling clay with the thumb and to let a minute drop of ether run down inside. When this has been done, the tube is removed, and the contents are tipped out into a petri dish or similar container, ready to be mounted. The specimens are best collected with the aid of a fine brush which is moistened in water.

A mite or similar form which is to be mounted is selected, picked up on the tip of the brush, and transferred to a drop of whichever of the two gum media has been selected. As little water as possible should be transferred with it, and the mite should be pushed under the surface of the gum with the point of a needle. The mount is inspected under the low power of the microscope, and, if any large quantity of air has been carried in with the mite, the bubbles are released with the aid of a fine needle and allowed to come to the surface before the coverslip is laid gently into place. The drop of gum should be large and no endeavor should be made to press the coverslip down. If a reasonably thick layer of mountant is left, almost any small arthropod will spread its legs like a textbook diagram before dying and will remain in this form indefinitely. As soon, therefore, as each mount has been made, it is pushed aside, and the next specimen is mounted, so that in a remarkably short space of time one has permanent records of all the small arthropods which were present. The finishing of these slides has already been discussed and may be carried out at any time after they have been made.

Summary

1. Erect Berlese funnel.
2. Transfer small arthropods to drop of mounting medium on slide.
3. Add coverslip.

Example 2

Preparation of a Wholemount of Pectinatella Stained in Grenacher's Alcoholic Borax Carmine

Though this description applies to the animal named, it may be used equally well for any other fresh-water bryozoan or, as a matter of fact, for any small invertebrate of about the same size and consistency. Pectinatella has been picked only for the reason that it has the habit of turning up on the walls of the aquaria in the author's laboratory. Profitable hunting grounds, if they have to be sought, are the undersides of the leaves of large water plants and the surfaces of branches of trees which have fallen into the water but have not yet had time to decay. An old trick of European collectors was to lower a length of rope into a pond in which Bryozoa were known to occur and to leave it there for the summer. It was astonishing how frequently, when these ropes were pulled up again in the fall, they were found to be covered with colonies of Bryozoa.

However the Bryozoa are obtained, it is necessary first that they be narcotized. The material on which they are living is cut up and placed on the bottom of a finger bowl of aquarium or pond water. Distilled water and tap water are lethal to these forms. There should not be so many specimens that they touch each other on the bottom of the finger bowl, and the finger bowl itself should be completely filled with water. The fresh-water Bryozoa are a little sensitive to heat and may not respond well to the high temperatures found in some laboratories. In this case it is well to put the finger bowl containing the specimens in an icebox or electric refrigerator, preferably one held at about 10° C., and to leave them there overnight. Then they may be brought out and narcotized before they have time to suffer from the increasing temperature.

The author prefers to use menthol, which is both cheap and easy to obtain. The menthol is sprinkled on the top of the water in the specimen jar. For an ordinary finger bowl, about a gram of menthol will be sufficient. There is no means of foretelling how long it will take the specimens to become narcotized; therefore, they should be observed at intervals until they no longer are seen to be contracting. However this may not be due to narcotization, so that some very delicate instrument—a hair mounted in a wooden handle is excellent—should be used to test narcotization by pushing the individual polyps. If, on receiving a push, they contract sharply, it is evident that no narcotization at all has taken place, and the amount of menthol which has been sprinkled on the surface should be increased greatly. If, on being pushed with a hair, they contract slowly, it is evident that they are partly narcotized. One must be exceedingly careful not to disturb them further for at least 10 minutes, for if they contract in a narcotized condition they will not expand again. The right stage for killing has arrived when no amount of shoving with a hair will persuade the specimens to contract and an examination under a binocular microscope shows that the ciliary action on the lophophore has not stopped. A rubber tube is used to siphon carefully from the finger bowl enough water so that the remaining layer just covers the specimens. Then the finger bowl is filled with 4 per cent formaldehyde, covered, and put to one side.

One must be very careful to distinguish between a "killing" agent, as formaldehyde, and "hardening" and "fixing" agents. In the present instance it is quite unnecessary, since a stain containing in itself an adequate mordant is to be used, to employ any fixative which will combine with the proteins of the specimens, but it is necessary to harden them, in order that they may withstand the treatment to which they will be subjected in staining and dehydration. Four per cent formaldehyde hardens very slowly, and it is suggested that next the specimens be passed to alcohol for the hardening process.

It is desirable, however, that they be flattened, before hardening, into the shape that they will be required to assume after mounting. It is to be presumed that the purpose of making a microscope slide is to study the object which has been mounted; the depth of focus of microscope lenses is so slight that only relatively thin objects can be studied. It is extraordinary how frequently this simple principle is overlooked or how frequently people endeavor to flatten the object after it has been mounted in balsam and is almost invariably so brittle that it will break up during the flattening process. Five minutes' work in arranging the parts before hardening makes all the difference between a first-class and a second-class mount. To arrange and flatten the objects for hardening, the 4 per cent formaldehyde is replaced with water—a matter of convenience—and then the first specimen to be treated is selected. This specimen is removed to a finger bowl of clean distilled water where it is examined thoroughly to make sure it has no adherent dirt. The object is flattened by hardening it between two slides, but it will be obvious that if it is just pressed between two slides it will be squashed rather than flattened. Anything may be used to hold the two slides apart, though in the present instance a very thick No. 3 or two No. 2 coverslips would give about the right separation. Therefore, at about an inch on each side of the center of a glass slide is placed a thick No. 3 coverslip, which may be held in place by the capillary attraction of a drop of water. The specimen is picked up from the water with a large eye-dropper type pipette and placed in a large volume of water on the slide. It is then easy to arrange the parts with needles, but it is difficult to lower a second slide without disarranging these parts. An alternative method is to place the slide with its coverslips in the finger bowl with the specimen, to arrange its parts under water, and to place the second slide on top. Whichever process is adopted, the slides are tied or clipped together and transferred to a jar of 96 per cent alcohol where they may remain for a week or until next required. Each specimen is treated in this manner. It is better not to try to flatten two or three specimens on one slide.

When it is time to continue mounting the specimens, the slides are placed in a finger bowl of 96 per cent alcohol before cutting the cords or removing the clips which bind them together. Getting the two slides apart without damaging the specimen is not easy, particularly if the specimen tends to stick to one or the other of the slides. The simplest method is to insert the blade of a scalpel into the gap between the slides and twist it slightly to see whether or not the specimen is free. If the specimen shows signs of sticking to one slide, the other may be removed, and the specimen washed from the slide to which it is stuck with a jet of 96 per cent alcohol from a pipette. If it shows signs of sticking to both slides, it is still possible to free it from both by projecting a jet of 96 per cent alcohol between them. Each slide is treated in due order until one has accumulated all the flattened specimens in a dish of 96 per cent alcohol. It must be understood that these specimens have been hardened flat, so that no amount of subsequent treatment will ever swell them out again or prevent them from remaining in the required position.

It is recommended, if one has several specimens to handle, that a series of the little cloth-ended tubes shown in Figure 9 be used. The only alternative is to handle each specimen with the aid of a section lifter with the consequent risk of damage. Though not nearly so satisfactory, it is also possible at least for the process of staining and dehydration, to place all the specimens in a small vial in which the different fluids used may be placed successively.

A wholemount of this type is best stained in carmine, and the choice would lie between Mayer's carmalum and Grenacher's alcoholic borax carmine; the author's preference is for the latter. The preparation of the latter stain, the formula for which is given in Chapter 2, does not present any difficulty, but it should be noted that a differentiating solution of 0.1 per cent hydrochloric acid in 70 per cent alcohol will be required. Adequate supplies of this should be available before one starts staining.

The specimens are now passed from 96 per cent alcohol to 70 per cent alcohol. Naturally, they will float, but, as soon as they have sunk to the bottom, it may be presumed that they are sufficiently rehydrated. Either the cloth-ended tube containing them may be transferred to the dish of stain or the 70 per cent alcohol may be poured out of this tube and stain substituted for it. Two of the advantages of this stain are that it is relatively rapid in action—very few specimens will not be stained adequately in 5 to 10 minutes—and it does not matter how long the materials remain in it. It is, therefore, often convenient to leave the specimens in stain overnight and to start differentiation the next morning. Either they are removed to differentiating solution or, alternatively, the stain is poured off and the differentiating solution substituted for it. In the latter case three or four changes will be required, owing to the necessity of leaving some stain in the bottom of the tube to avoid pouring the specimens out with it. Indeed, unless the operator is quite experienced, it is safer to shake the tube, so as to distribute the specimens thoroughly in the stain, and then to tip everything into a large finger bowl of differentiating solution from which the specimens may be picked out later and transferred to a new tube of differentiator. It is tragically easy, in pouring off the stain, to pour specimens with it down the sink. As soon as the stain has been washed off with the differentiating solution, a single specimen should be transferred to a watch glass and examined under a low power of the microscope. It is more than probable that little differentiation will be required, so that a simple rinse may be adequate. It is difficult to judge the exact degree of differentiation required, and it must be remembered that the object will appear darker after clearing than it does in the differentiating solution. The internal organs should be sharply demarcated when the outer surfaces of the specimen are relatively free from stain. This may be judged in Pectinatella by placing a coverslip on the specimen and examining one of the branches of the lophophore under the high power of the microscope. Differentiation may be considered complete when only the nuclei in the cells of the lophophore are stained. The specimens are washed in four or five changes of 70 per cent alcohol to remove the acid before they are placed for at least a day in 96 per cent alcohol as the first stage of dehydration. Next they should be transferred to two changes of at least six hours each in a considerable volume of fresh 96 per cent alcohol and cleared. Absolute alcohol is not necessary if one is using terpineol as a clearing agent.

There is some danger, if specimens are transferred directly from 96 per cent alcohol to a fluid as viscous as terpineol, that they will become distorted through the violent diffusion currents. This may be readily avoided in the following manner. A fairly wide (about an inch) glass vial is filled about half-full with terpineol. Ninety-six per cent alcohol is poured very carefully down the side of the vial (or on a spoon held in the vial in the manner of a bartender making a pousse-café) in order to float a layer of alcohol on top of the terpineol. The specimens are now dropped into the alcohol. Naturally they sink through it, coming to rest on the surface of the layer of terpineol into which they sink slowly without any strong diffusion currents. They will be seen to have sunk to the bottom after a little while, but there will still be a quantity of alcohol diffusing upward from them. As soon as these diffusion currents have ceased, the alcohol should be drawn from the top of the tube with a pipette and the specimens transferred to clean terpineol. When they are in fresh terpineol, they should be examined carefully under a microscope to make sure that they are glass-clear without the least trace of milkiness. If they appear slightly milky, they have either been dehydrated insufficiently or the alcohol used for the preparation has become contaminated with water. In either case they must be transferred to a tube of fresh alcohol for complete dehydration. Then they are transferred back into terpineol in the manner described. It is a sheer waste of time to endeavor to prepare a balsam mount from a specimen which is not perfectly transparent in the clearing medium.

When all the specimens are in natural balsam, one should have ready some clean slides, some clean ¾-in. circular coverslips, and a balsam bottle containing natural balsam. The author's preference for the natural balsam rather than a solution of this material in some solvent has been explained previously (see p. 46). For example, a drop of the natural balsam is placed on each of six slides, and then, one at a time, six specimens are lifted from the terpineol and placed on top of the balsam. The specimens will sink through the balsam very slowly, so that these six slides should be pushed aside while the next six slides have drops of balsam put on them, and so on. As soon as a specimen has sunk to the bottom of the drop of balsam, a coverslip is held horizontally above, touched to the top of the drop, and then pushed down with a needle until the specimen is flattened firmly against the slide. Since these specimens have been properly hardened and flattened, there is no risk of their being damaged by drying the mount under pressure, so that a clip can be applied (see Fig. 12) and the slides placed on a warm table to harden. Each is cleaned, finished, and labeled in the manner suggested previously (see p. 75).

Summary

1. Narcotize with menthol.
2. Kill with 4 per cent formaldehyde.
3. Harden selected polyps compressed between slides in 95 per cent alcohol.
4. Stain 1 to 24 hours in Grenacher's alcoholic borax carmine.
5. Differentiate in acid 70 per cent alcohol until pink.
6. Dehydrate, clear in terpineol, and mount in balsam.

Example 3

Preparation of a Wholemount of a 48-hour Chick Embryo, Using the Alum Hematoxylin Stain of Carazzi

Fertile eggs are relatively easy to secure and should be incubated at a temperature of 103° F. for the required period of time. The term "48-hour chick" is relatively meaningless since the exact stage and development which will have been reached after two days in the incubator depends on the temperature of the latter, on the temperature at which the egg was stored prior to its incubation, and even on the age of the hen. It is desirable therefore, if any very specific age of development is required, to start a series of eggs in the incubator at three- or four-hour intervals and then to fix and mount these at the same time.

For the removal of the embryos from the egg, one needs first a series of finger bowls or any circular glass dishes of 5 to 6 in. in diameter and 2 to 3 in. in depth, a number of syracuse watch glasses, a large quantity of a 0.9 per cent solution of sodium chloride, a pair of large dissecting scissors, fairly fine forceps, a pipette of the eye-dropper type, some coarse filter paper, and a pencil.

No very great accuracy is required in the making up of the normal salt solution, and any percentage between 0.7 and 1 will be sufficient for the purpose in mind. Though it is customarily specified that the temperature of the solution should be 102 to 103° F., anywhere within 10° on either side of these figures is relatively safe. The egg is removed from the incubator and placed in one of the finger bowls, which is filled with the warm normal saline solution until the egg is totally immersed. If the operator is rather skilled, it is possible, of course, just to break the egg into the warm saline solution as though one were breaking it into a frying pan, but it is recommended that the inexperienced prepare several hundred wholemounts before they endeavor to do this. The method by which the inexperienced can be assured of securing a perfect embryo on every occasion is first to crack open the air space which lies at the large end of the egg and to let the air bubble out through the warm saline solution. This permits the yolk to fall down out of contact with the upper surface of the shell, which may be removed with blunt-nosed forceps, working from the air space toward the center. Again the matter of practice is involved, for a skilled operator can remove this shell in large portions, while the inexperienced should work very carefully to avoid puncturing the yolk. If the yolk is punctured, it is much simpler to throw the egg away and start with another one. After about half the shell has been removed, it will be found quite easy to tip the yolk, with the embryo lying on top of it, out into the saline solution and remove the shell.

The next operation is to cut the embryo from the yolk by a series of cuts made well outside the terminal blood vessel, which marks the limits of the developing embryonic structures. To do this with success requires more courage than experience. Just as soon as the vitelline membrane is punctured, the yolk starts squirting out through the hole, rendering the fluid milky so that one can no longer see the embryo. The smaller the hole which is cut, the more violently does the yolk squirt out, so that the larger the scissors which can be employed, the more easily will the embryo be removed. The easiest method is to take blunt forceps in the left hand and with it grip the extra-embryonic areas of the chick well outside the sinus terminalis. A certain amount of drag is placed on it so that the vitelline membrane is wrinkled, and then with a large pair of scissors a transverse cut is made directly away from the operator about a third of an inch outside the sinus terminalis on the side of the embryo opposite that which is held by the forceps. This initial cut should be at least an inch long and should be made firmly. Two cuts at right angles to the first, each an inch in length, should be run on each side of the embryo. The part gripped with the forceps should be released, and the free edge, where the first cut was made, gripped so that the embryo can be folded back away from the yolk. It is now relatively easy to sever all connection between the embryo and the underlying materials by a fourth cut. The embryo, held by the forceps in the left hand, will now be floating free in the saline solution. The embryo is much stronger than it looks and will not be damaged provided the tips of the forceps are kept under the saline solution.

The embryo must be transferred to clean saline solution, preferably in another finger bowl. This transfer may be made either with a very wide-mouthed pipette of the eye-dropper type or by scooping it up in a smaller watch glass with plenty of saline solution and transferring it to the fresh solution. Here it should be picked up again by one corner with the forceps and waved gently backward and forward to remove the adherent vitelline membrane (which may have fallen off already) as well as such yolk as remains. At this stage the embryo should be examined to make sure that the heart is beating and that it is in fit condition for fixation.

The embryo is now scooped out on one of the syracuse watch glasses with as little water as possible. Next it is necessary to persuade it to flatten on the bottom in an upside-down position; that is, so that the portion of the embryo which was previously in contact with the yolk is now directed toward the operator. To determine which side of the embryo is uppermost requires considerable practice unless the primary curvature of the head toward the right has already started. The best point of examination is the heart, which lies, of course, on the lower surface of the embryo. Having maneuvered the embryo in the saline solution in the watch glass until it is in the upside-down position required, the water should be drained off with the aid of a pipette, which is run rapidly with a circular motion around the outside of the blastoderm while the water is drawn up. As experience will soon show, any attempt to drain the water up a stationary pipette will result in the embryo being drawn out in the direction in which the water is being sucked. A little practice in running the pipette round and round the outside of the blastoderm and about a millimeter away from it will enable the operator to strand the embryo perfectly stretched in all directions. Under no circumstances whatever should a needle be used in an endeavor to arrange the embryo because the point will adhere to the blastoderm from which it cannot be detached without damage. If the embryo is not flattened and spread out satisfactorily, it is necessary only to add a little clean saline solution with a pipette and repeat the operation.

A piece of coarse filter paper or paper toweling is cut into a rectangle of such size that it will drop easily into a syracuse watch glass. An oval or circular hole is cut in the middle of this (done most easily by bending it in two and cutting a semicircle) of such a size as will cover exactly those areas of the embryo which are to be retained. That is, if the embryo alone is required, the hole may be relatively small, while if it is desired to retain all the area vasculosa with its sinus terminalis, the hole must be correspondingly enlarged. The hole must not be larger, however, than the blastoderm removed from the egg because the next operation is to cause the unwanted extraembryonic regions to adhere to the paper, leaving the embryo clear in the center. By this means alone will the embryo be prevented from contracting and distorting when fixative is applied to it. Such data as are pertinent may be written on the edge of the paper rectangle in pencil. Then the paper is dipped in clean saline solution. If the saline used has already become contaminated with egg white, a sharp puff of air should be directed at the hole to make quite certain that a film of moisture does not extend across it since the bubbles so produced always disrupt the embryo if this film is left. Now the rectangle of filter paper is dropped on top of the stretched embryo in such a manner that the embryo does not become distorted. This is, in point of fact, a great deal easier than it sounds although a few false trials may be made by the beginner. The author's procedure is to place one end of the rectangle on the edge of the watch glass nearest him, taking care that it does not touch the blastoderm, and then to let the paper down sharply. The edges of the blastoderm must be in contact with at least two thirds of the periphery of the hole if it is to remain stretched. As soon as the paper has been let down, the end of a pipette or a needle should be used to press lightly on the edges of the paper, where it is in contact with the blastoderm, to make sure that it will adhere.

The embryo is now ready for fixing. The choice of a fixative, naturally, must be left to the discretion of the operator. The author's preference, where hematoxylin is to be employed for staining, is for a mercuric mixture, such as the solution of Gilson (see p. 10). If much embryonic work is to be undertaken, reference should be made to Gray's *Microtechnique* for the formula of Gerhardt's fixative, which is better. The disadvantage of the customarily-used, picric acid formulae is that they interfere seriously with subsequent staining by hematoxylin. The fixative should be applied from an eye-dropper type pipette in the following manner. First, a few drops are placed on the center of embryo, so that a thin film of fixative is spread over it. After a moment or two a little more may be added with a circular motion on the paper which surrounds the embryo. Again the paper should be pressed on the periphery of the blastoderm with a needle or the end of the pipette to make sure that the adherence is perfect, and the whole should be left for a moment or two before being shaken gently from side to side to make quite certain that the embryo is not sticking to the watch glass. If it is sticking, the end of the pipette containing the fixative should be slid under the edge of the paper, and a very gentle jet of fixative used to free the embryo. As soon as the embryo is floating freely in fixative, the syracuse watch glass may be filled up with fixative and placed to one side, while the same cycle of events is repeated with the next embryo. After about 10 minutes in the fixative, the paper may be picked up by one corner and moved from reagent to reagent without the slightest risk of the embryo becoming either detached or damaged. Care, of course, must be taken not to pick up the paper with metal forceps unless the instrument has been waxed first because the mercuric chloride in the fixative will damage the metal. It is the author's custom to leave the embryos in the watch glasses for about 30 minutes before picking them out and transferring them to a large jar of the fixative, preferably kept in a dark cupboard. The total time of fixation is not important but should be not less than one day nor more than one week. When the embryos are removed from the fixative, they should be washed in running water overnight and may then be stored in 70 per cent alcohol for an indefinite period.

When one is ready to stain a batch of embryos, it is necessary only to take them from 70 per cent alcohol back to distilled water until they are thoroughly rehydrated, and then transfer them to a reasonably large volume of Carazzi's hematoxylin where they may remain overnight. The gravest mistake which can be made in this type of staining is to stain initially for too short a period.

The result is that the outer surface of the embryo becomes adequately stained while the inner structures do not, but this defect is difficult to detect until the embryo is finally cleared for mounting. When the embryos are removed from the stain, at which time they should appear a deep purple, they should be transferred to a large finger bowl of distilled water, where they are rocked gently backward and forward until most of the stain has been removed from the paper to which they are attached. Each embryo should then be taken separately and placed in 0.1 per cent hydrochloric acid in 70 per cent alcohol. The color will immediately start to change from a deep purple to a pale bluish pink, and the embryos should remain in this solution until, on examination under a low power of the microscope, all the required internal structures appear clearly differentiated. Most people differentiate too little, forgetting that the pale pink of the embryo will be changed back to a deep blue by subsequent treatment and that the apparent color will also increase in density when the embryo is cleared. No specific directions for the extent of the differentiation can be given beyond the general advice to differentiate far more than you would anticipate to be necessary. After the embryos have been sufficiently differentiated, each one should be placed in alkaline tap water, either as it occurs in nature or rendered alkaline with the addition of sodium bicarbonate. Here it should remain until all the acid has been neutralized and the embryo itself has been changed from a pink back to a deep blue coloration. It may then be dehydrated in the ordinary manner through successive alcohols. It is the author's custom to remove it from its paper only when it is in the last alcohol and before placing it in the clearing agent. Some persons place it in the clearing agent attached to its paper and remove it only before mounting. Any clearing reagent may be tried at the choice of the operator; the author's preference for chick embryos is terpineol which has the advantage of not rendering these delicate structures as brittle as do many other reagents. The cleared embryo is mounted in balsam, preferably with the coverslip supported in the manner described on page 41.

Summary

1. Incubate fertile eggs at 103° F. for 48 hours.
2. Remove shell under surface of warm 0.9 per cent sodium chloride.
3. Detach embryo from surface of yolk with bold cuts and wash in clean saline solution.
4. Strand embryo in watch glass.
5. Cut hole slightly larger than embryo in filter-paper rectangle, soak paper and drop it over embryo.
6. Fix in Gilson's fluid 1 to 6 days.

7. Wash in running water overnight.
8. Stain overnight in Carazzi's hematoxylin.
9. Differentiate in acid 70 per cent alcohol until internal anatomy is shown clearly.
10. Treat with alkaline tap water until blue.
11. Dehydrate.
12. Detach embryo from paper and clear in terpineol.
13. Mount in balsam.

Example 4

Preparation of a Wholemount of a Liver Fluke, Using the Carmalum Stain of Mayer

Though many persons will be forced to rely on a supply house for their material, much better preparations can be made if the living flukes are secured from a slaughter house. In this case, the flukes should be removed from the liver, where many will be found crawling upon the surface if the animal has been dead for some time, to a thermos flask containing physiological saline solution at a temperature of about 35° C., to which has been added a small quantity (approximately 0.1 Gm. per liter) of gelatin. Flukes can be transported alive for relatively long distances in this solution, and every possible effort should be made to keep them alive until they have been brought to the laboratory and are ready to be fixed. In the laboratory the contents of the thermos flask should be poured into a dish, and the worms transferred individually to another large dish containing warm physiological saline where the last of the blood will be washed from them. Better preparations will be secured if time is taken to anesthetize the worms before fixing them, since most of the thick and opaque mounts which one sees in laboratories result from an endeavor to fix an unanesthetized worm which has contracted during the course of fixation. Liver flukes are easy to anesthetize; the simplest method is to sprinkle a few crystals of menthol on the surface of the warm saline solution and leave the flukes in this for about half an hour. Of course, one should not permit the worms to die in this solution but should watch them carefully, terminating the process when their motions are exceedingly slow and consist only of occasional feeble contractions rather than the active movements in which they were indulging when removed from the liver.

While the worms are being anesthetized, preparations for fixing them should be made. Take two sheets of quarter-inch plate glass, large enough so that the number of worms which are to be fixed can be laid on them, and place upon the lower plate two or three thicknesses of a rather coarse filter paper or paper toweling. Blotting paper is too soft to be used for the purpose, and a good filter paper is much to be preferred to a paper towel. The selection of fixative must rest, of course, in the hands of the operator, but the author's preference is for the mercuric-acetic-nitric mixture of Gilson. This has all the advantages in sharpness of definition given by mercuric fixatives, while the addition of nitric acid appears to render the flattened worms less brittle in subsequent handling. Whatever fixative is selected, the sheet of filter paper is saturated thoroughly with it, and the anesthetized worms removed from the physiological saline and laid out one by one about an inch apart on the paper. This must be done as rapidly as possible to prevent fixation from taking place before an additional layer of paper is placed on top, saturated with fixative, and the second sheet of glass placed on top of this. Assuming that the sheets of glass employed are of the size of a sheet of typewriting paper, it is suggested that about a two-pound weight be placed on the upper sheet of glass. The whole should now be left for at least 12 hours before removing the glass and upper paper, picking the worms up one by one on a glass section lifter (metal cannot be used because of the presence of mercuric chloride), and transferring them to a large jar of fixative where they may remain for another day to another week at the discretion of the technician.

At the conclusion of fixation the worms should be washed in running water for at least 24 hours. It has not been the author's experience that this fixative, followed by such washing, requires the use of iodine for the final removal of the mercury. At this stage, moreover, iodine tends to render the worms brittle, and the author would strongly recommend its omission. After thoroughly washing in water, the worms may be stained; the formula selected for the purpose of this example is the well-known carmalum of Mayer. Objects of this type are better stained by the additive process than by a process of differentiation; that is, they are better placed in an exceedingly weak solution and allowed to absorb the stain slowly than placed in a strong solution which requires subsequent differentiation. The best diluent for the stain is a 5 per cent solution of potassium alum. The extent of the dilution is dependent upon the choice of the operator and the size of the object which is to be stained. In the present instance a dilution of about 1 part of the stain to 100 parts of 5 per cent potassium alum would be correct. It is far more dangerous to have the solution too strong than it is to have it too weak, and, since it is an excellent preservative, the worms can remain in it for an indefinite period. The worms are merely placed in this diluted stain and left there until their internal structures have become clearly visible. It is suggested that they be examined at the end of a week, and subsequently every three days, until such time as examination with a low-power binocular microscope, using a bright light from beneath, shows the testes to be darkly stained. At this point the worms are removed to a fresh clean solution of 5 per cent potassium alum and rinsed for a short time to remove all the adherent color. However, they will still be pink on the outside. Since the purpose of the stain is to demonstrate the internal organs, it is obviously desirable to bleach this outer layer in order to produce bright scarlet internal organs against a white background. In the experience of the author this may be done most readily with the aid of a potassium-permanganate, oxalic-acid bleach in the following manner. Prepare a solution of potassium permanganate so weak that it appears only a very faint pink. This is best done by adding a few drops of a strong solution to a beaker of distilled water. Then each worm is dropped individually into the solution and allowed to remain there until such time as it has turned a bronzy brown on the outside. This appearance can best be detected in reflected light. Just as soon as the first bronze sheen appears on the outside of the worm, it must be removed to fresh distilled water where it can remain until all the other worms in the batch have been treated similarly. It will be necessary to renew the potassium permanganate from time to time, which is done by adding a few more drops of the stock solution to the beaker. The strength of oxalic acid which is used to bleach the worm is quite immaterial; 2 or 3 per cent, arrived at by guess work rather than by weighing, is an adequate solution. Since the bleaching of the surface is not nearly as critical as is the deposition of the potassium permanganate, all the worms may be bleached at the same time by pouring off the distilled water and substituting the oxalic acid for it in the beaker. One or two twists of the wrist to rotate the worms in the beaker will result in their turning from a bronze sheen to a dead white. The oxalic acid is poured off without any waste of time, and the worms are washed in running tap water for an hour or so before being dehydrated in the ordinary way, cleared, and mounted in balsam. Little trouble will be experienced with curling of the worms if they have been fixed and treated as described. If they should curl, they should undergo the final dehydration in 96 per cent alcohol while being pressed loosely between two sheets of glass, which will be sufficient to hold them flat.

Summary

1. Anesthetize flukes in warm saline solution with menthol.
2. Place a layer of filter paper saturated with Gilson's fluid on a glass plate. Have ready another sheet of fixative-saturated paper and another glass plate.
3. Lay worms about an inch apart on paper on glass, cover with second sheet saturated with fixative, lay second glass on top, and add sufficient weight to flatten but not squash worms.
4. After about 12 hours remove flattened worms to jar of fixative. Fix 1 to 6 days. Wash in running water overnight.
5. Transfer to 1 part Mayer's carmalum diluted with 100 parts 5 per cent potassium alum. Leave until internal structures are stained clearly (1 to 3 weeks).
6. Wash in 5 per cent potassium alum.
7. Bleach surface with permanganate-oxalic acid.
8. Wash thoroughly, dehydrate, clear, and mount.

Example 5

Smear Preparation of Monocystis from the Seminal Vesicle of an Earthworm

Very few Sporozoa are available for class demonstration purposes, and one's choice is practically limited to the inhabitants of the intestines of a cockroach or to the specimen under discussion.

The advantage of using Monocystis is that all the forms from the sporozoite to the trophozoite occur in the seminal vesicle of the earthworm and, therefore, may be made available on a single smear. The degree of infection among earthworms varies greatly, but it has been the author's experience that the larger the earthworm, the more likely the chance of a heavy infestation. At the same time, there is no use making a great number of smears for class demonstration purposes until a preliminary survey of a single smear has shown that the material will be satisfactory.

There is no need to kill or anesthetize the earthworm, which is simply pinned down in a dissecting tray and slit from the anterior end to about the sixteenth or seventeenth segment. The edges of this slit are pulled back and pinned into place, disclosing the large white seminal vesicles.

One should have available, before making the smear, a petri dish in which are a couple of short lengths of glass rod, a supply of the fixative selected, an adequate supply of clean glass slides, an eye-dropper type pipette, some 0.8 per cent sodium chloride, and some coplin jars of distilled water. Enough fixative is poured into the petri dish so that when a slide is laid on the pair of glass rods its lower but *not* its upper surface will be in contact with the fixative. This level should be established with a plain glass slide before the smears are made.

Now the seminal vesicle of the earthworm is slit, and a drop of the contained fluid is removed with the eye-dropper type pipette. This pipette is used to smear a relatively thick layer of the material on the center of one of the clean slides. Before it has time to dry, this slide is laid face down in the fixative for a period of about two minutes. Then the slide is removed, rinsed under the tap, and examined under a high power of the microscope after a coverslip has been placed over the smear. It is rather difficult to see the trophozoite stages in an unstained preparation, but no difficulty will be experienced in picking out the spore cases (pseudonavicellae) because of their relatively high index of refraction. It may be assumed that adequate numbers of the parasites are present if not less than three or four of these spore cases occur within the field of a four-millimeter objective in a thick smear of this nature.

If the worm shows satisfactory infection, the remainder of the material from the eye-dropper pipette is placed in a watch glass and diluted with 0.8 per cent sodium chloride until it forms a dispersion about intermediate in thickness between cream and milk. Working as rapidly as possible, as many smears as are required are made from this dilution. The dilution in question will not retain the parasites in good condition for more than about five minutes, but, if insufficient smears have been made in this time, it is easy to take a fresh supply of the seminal fluid from another vesicle and to dilute it in the fresh watch glass. The smears should be made with two slides in the manner described in Chapter 7, and each slide should be placed face downward in fixative for three or four minutes before being removed to a coplin jar of distilled water.

After they have been washed in water, the smears should be transferred to 70 per cent alcohol where they can remain until they are ready for staining. Any stain may be used, but it is conventional to employ a hematoxylin mixture. The method of staining is easy. The solution is made in accordance with the directions given and diluted to the extent of about 2 per cent with distilled water. The slides are placed in this diluted solution and left until examination under the low power of the microscope shows them to have been adequately stained and differentiated. Then they are rinsed exceedingly briefly in distilled water and dried. There is not the slightest necessity to employ any dehydrating agent, such as alcohol, for the smears should be sufficiently thin and the objects in them sufficiently well fixed that drying will not make the least difference. To complete the mount, a drop of the mountant selected is placed in the center of each smear, a coverslip added, and the whole put on a warm plate until it is dry.

Summary

1. Make a temporary, thick smear from the seminal vesicle of an earthworm. If plenty of spore cases are present, proceed with step 2; if not, test other worms until a heavily infected specimen is found.
2. Place two glass rods in a petri dish and lay a slide across them. Pour in just enough fixative to wet the lower surface of the slide.
3. Dilute the contents of the infected seminal vesicle with 0.8 per cent sodium chloride to the consistency of a thin cream.
4. Make a smear of cream as described on page 49.
5. Lay each smear face down on glass rods in fixative for 3 to 4 minutes. Wash and accumulate in a coplin jar of 70 per cent alcohol.
6. Stain and differentiate smears. Dry in air. Mount in balsam.

Example 6

Staining a Bacterial Film with Crystal Violet by the Technique of Lillie

This technique is so simple that it would be scarcely worth the trouble to describe it were it not necessary for the benefit of those who have never previously handled bacterial material and who may wish to attempt this for the first time.

The only tools and reagents necessary are a clean glass slide, a wire loop of the type used normally in bacteriology, a drop bottle containing the crystal violet stain, and a wash bottle containing distilled water.

The freshly flamed wire loop is touched as lightly as possible either to the surface of the medium in a test tube, or to the surface of the colony in an agar petri-dish culture. Then the loop is touched lightly to the center of the clean slide to transfer the bacteria. The only mistakes likely to be made by the beginner are in securing too great a quantity of material or in making too large an area on the slide. It must be remembered that the specimen is to be examined under an oil-immersion lens, so that the smallest possible smear derived just by touching the slide with the moist platinum loop will have an ample area for the purpose required.

If the microörganisms have been taken from a liquid culture which has not yet reached a very thick stage of growth, the spot on the slide now may be permitted to dry in air; but if the bacteria have been taken from a colony on the surface of a dish, it is necessary to dilute the drop on the slide. For this purpose, a small quantity of water in the same platinum loop is touched to the same spot, rubbed backward or forward once or twice, and then the slide permitted to dry.

As soon as the slide has dried, which should only be a moment or two if a sufficiently small quantity of suspension of the specimen has been used, it is taken and "heat fixed" in the flame of a bunsen burner or a spirit lamp. This is done by passing the slide quite rapidly twice through the flame. The actual temperature should not exceed about 80° C., and it is customary to hold the slide smear downward as it passes through the flame. Care must be taken that the slide is dried before it is thus quickly flamed or, of course, the bacteria will burst and be worthless.

On the flamed and dried smear is placed a drop of the selected stain, leaving this in place for about 30 seconds. The time is not critical, and any time between a half and one minute is perfectly satisfactory. It will be noticed that the stain frequently evaporates slightly, leaving a greenish film on the surface, and therefore, it is much better to wash it off with a jet from a wash bottle than to endeavor to rinse it off. This jet should be directed from the fine orifice of the wash bottle at an angle of about 30 degrees to the slide and should be intended to hit the edge of the drop. The jet will instantly lift off and float away the surface film, as well as wash excess stain out of the preparation. The preparation is now permitted to dry. When dry, it may be touched with immersion oil and examined under the oil-immersion lens. If it is desired to maintain this specimen permanently, it may also have a drop of Canada balsam placed on top of it and a very thin coverslip pressed into position. These specimens, however, are so easy to prepare that it is rarely necessary to preserve them permanently. The dried smear, if kept free from dust, may be preserved for as long as a year without further preparation.

Summary

1. Use a wire loop to make a small, thin smear of a diluted bacterial culture on a clean slide.
2. Dry smear in air, then pass it two or three times through a flame.
3. Place a drop of crystal violet stain on the smear, allow it to remain for 30 seconds, and wash off with a jet of water from a wash bottle.
4. Dry smear without heat.

Example 7

Demonstration of Gram Positive Bacteria in Smear Preparation by the Method of Gram

For the benefit of those who are not acquainted with bacteriology, it may be said by way of introduction that it has been customary since the time of Gram to utilize the reactions of bacteria to iodine mordanting as a basis of diagnostic classification. All bacteria, without reference to their nature, may be stained by the method given in Example 6, but there are some bacteria from which this stain can be removed by the action of an iodine-potassium-iodide solution reinforced with alcohol. The bacteria from which the stain is not removed are known as Gram positive; those from which the stain is removed are known as Gram negative. The solutions required are the violet used in Example 6 and Gram's iodine solution, the formula for which is given on page 12. Iodine is very soluble in strong solutions of potassium iodide but is only slightly soluble in weak solutions. If the total quantities of iodine and potassium iodide are placed in the total quantity of water, a period of as long as a week may elapse before a solution is complete; if the dry iodine and the dry potassium iodide are mixed together and a few drops of water added, the whole will go almost instantly into solution.

Presuming that one is working with a pure culture of bacteria, a smear is prepared as described in Example 6, taking the same precautions as to dilution mentioned there if the material is obtained from a bacterial colony. The smear is dried, flamed, and a drop of crystal violet poured on it from a drop bottle exactly as in the previous example. In this instance, however, it is not desirable to extract too much of the stain with water. After the stain has been acting for two minutes or so, it is not removed, as in Example 6, with a jet from a wash bottle, but the entire slide is rinsed rapidly in a fairly large volume of water and a drop or two of the iodine solution poured over it. If many slides are being stained, it is probably simpler to drop each slide into a coplin jar containing the iodine solution rather than to pour iodine on it. After the iodine has been permitted to act for a period of 1 minute, the whole slide is given a quick rinse to remove the excess iodine and then placed into absolute alcohol until no more color comes away; unless the film is very thick, this will appear to decolorize it completely. It is passed from alcohol to water, which instantly stops differentiation, and then dried. Varying types of bacteria require varying periods of differentiation, but it is best for the beginner to use absolute alcohol until no more color comes away rather than to endeavor to control the differentiation under the microscope.

Though this is a Gram preparation by the original technique, it is customary nowadays to provide a counterstain of a contrasting color to bring clearly into evidence any Gram negative organisms which may be mixed with Gram positive. A 1 per cent solution of safranin is widely employed, and in this case, the second red contrasting stain is allowed to act from 5 to 10 seconds, washed off with water, and the slide then dried and examined under an oil-immersion objective.

Summary

1. Follow steps 1 through 3 of Example 6 but allow stain to act for 2 minutes. Then wash it off under tap.
2. Treat stained smear with Gram's iodine for 1 minute. Rinse under tap and soak in absolute alcohol until no more color comes away.
3. If desired, counterstain 5 to 10 seconds in 1 per cent safranin and wash it off with water before drying.

Example 8

Demonstration of Tubercle Bacilli in Sputum by the Technique of Neelsen

When Neelsen published his original technique for the demonstration of tubercle bacilli, the standard magenta solution used for the staining of bacteria was that proposed in the previous year by Ziehl. As a result, Neelsen's technique was referred to as a modification of Ziehl and to this day, the hyphenated term "Ziehl-Neelsen" is applied to almost any method for the demonstration of tubercle bacilli in sputum regardless of the author of the technique.

It is proposed here to describe the original technique of Neelsen, leaving it to the technician to determine which of the many other methods given in Gray's *Microtechnique* can be more readily applicable to his problem. It may be said in favor of the technique of Neelsen that it gives a far better differentiation of tubercle bacilli than do some of the more recent methods which, although they give good preparations, tend to cause certain errors of diagnosis through the ability of other bacteria to withstand the lower concentration of acids employed today.

It is to be presumed that the sputum collected from the patient will have been placed at the disposal of the technician in the glass vessel in which it was secured. It should be examined to see whether or not any small yellowish particles exist in it, and if they do, one of these particles should be extracted carefully with a sterile bacteriological wire loop and utilized for the preparation. Even if no such particles are visible to the naked eye, it is possible that tubercle bacilli will be present, but due consideration should be given to some method of concentrating these bacilli before making the smear. The standard method of concentration is to hydrolyze the sputum to the extent necessary with the aid of a weak solution of potassium hypochlorite, which is known to be without action on tubercle bacilli. For a long time a proprietary compound known as antiformin, which is a strongly alkaline solution of sodium hypochlorite, has also been employed for the same purpose. About an equal quantity of the selected solution and the sputum are placed in a centrifuge tube. The tube is incubated in a serological water bath for about 10 minutes and then centrifuged rapidly, the smear being made from the denser portions which remain at the bottom of the tube.

Whichever method is employed, the quantity of material removed by the sterile loop should be about the size of a large pinhead. That is, a great deal more material should be employed than in the preparation of a simple bacterial smear from a known culture for the reason that large areas have to be searched in the interests of diagnostic accuracy. This pinhead of material must be spread over the largest possible area of the slide, which is most readily accomplished by pressing another slide on the first and then drawing the two slides apart with a lateral motion. Both slides are dried in air and flamed as has been described in previous bacteriological preparations.

The solutions required for the original Neelsen technique are the carbol-magenta solution of Ziehl (see p. 22), a 25 per cent solution of nitric acid, and a 1 per cent methylene blue solution. The slide is first flooded with a large quantity of the magenta solution and then placed on a metal sheet, where it should be warmed underneath by a bunsen flame to the point where the stain is steaming briskly but no bubbles have appeared. If it shows signs of drying, fresh quantities of the magenta solution should be added to it. It either may be left at this temperature for 3 to 5 minutes or, as is customary in modern practice, it may be raised to steaming, permitted to cool, again raised to steaming, permitted to recool, and so on, until four such cycles have been completed. The slide is washed in a large volume of tap water until no further magenta comes away, and then placed in 25 per cent nitric acid until it is almost completely decolorized. It cannot be decolorized too far, but usually there will be found, even after prolonged exposure to the acid, a faint pink coloration of the background. Now the slide is washed in running water until all the acid is removed. Finally, it is treated with a blue stain for about two minutes to provide a contrasting coloration of any other bacteria present and washed thoroughly, dried, and examined in the customary manner.

It must be emphasized that this technique as described is designed specifically to show tubercle bacilli and is so violent that many bacteria which are acid-fast to less strong acids will be decolorized.

Summary

1. Smear yellow flecks or centrifuged concentrate from sputum between two slides. Separate, dry, flame, and stain both slides.
2. Flood smear with Ziehl's magenta, warm to steaming, and cool. Repeat warming and cooling three times.
3. Wash under tap and decolorize in 25 per cent nitric acid.
4. Wash off acid and counterstain, if desired, in 1 per cent methylene blue for two minutes.
5. Wash in water and dry.

Example 9

Preparation of a Transverse Section of a Root, Using the Acid Fuchsin-iodine Green Technique of Chamberlain

This is the simplest of all the preparations which is described in this part of the text, and can be recommended unhesitatingly to the beginner who has never previously prepared a section of any type. This preparation is designed to show only the skeletal outlines of the cells and is not intended to demonstrate in any way their cytological contents, which are removed in the course of preparation. If cytological detail in a botanical section is required, a paraffin section should be prepared and stained by the method given in Example 11.

It does not matter from what source the root is obtained, but it is recommended that the beginner select some soft root of about an eighth of an inch or somewhat less in diameter. If the root is collected from a living plant, it should be washed thoroughly to remove any adherent sand grains, which will spoil the edge of the cutting knife, and then preserved in 96 per cent alcohol until required. The 96 per cent alcohol should be changed as it becomes discolored, but with this precaution the specimens may be preserved indefinitely.

It is even possible to make preparations of this type from dried roots which have been preserved in a herbarium. The best method of swelling and softening these dried preparations is a 10 per cent solution of phenol in lactic acid. The lactic acid employed is the ordinary commercial solution in which the phenol should be dissolved immediately before it is required. Pieces of the dried root are placed in a reasonably large volume of this material and heated over a low flame to a temperature of about 50° C. Within 10 or 15 minutes a completely dried herbarium specimen will have become swollen out to its normal size and softened to the extent that sections may be cut readily from it.

The method of sectioning selected does not particularly matter. Since the sections cannot in any case be subjected to the first process while they are attached to the slide, there is no real advantage in embedding in paraffin and cutting in this medium if an ordinary hand microtome is available from which sections can be taken by the conventional method (see Chapter 8) of holding them either in pieces of pith or between the cut halves of a carrot. If the sections are cut by hand, they may be transferred immediately after they are cut to a dish of 20 per cent alcohol and from there to water; if they are cut in paraffin, they should be at least 20 microns in thickness. The ribbon, as it is removed from the microtome, should be dropped directly into a watch glass of xylene in which the paraffin will dissolve readily. The individual sections are then removed from the xylene with a section lifter, passed through absolute alcohol for the removal of the xylene, and thence graded down through alcohols to water. By whatever method the sections are produced, they are accumulated in a small dish of distilled water. These sections, of course, will retain the cell contents, which must be removed in order that the sections may be turned into true skeletons.

The best reagent to use for skeletonizing sections of plant tissue, is either potassium or sodium hypochlorite. Ordinary "bleaching solutions" sold for household purposes under various trade names are not suitable since they contain large quantities of calcium hypochlorite. If, however, the pure salts are not available, the household solution may be employed by adding to it enough of a solution of potassium or sodium carbonate to precipitate the calcareous contents and by filtering off the solution before use. If the pure salts are available, a 1 per cent solution may be employed conveniently. The sections are removed from the distilled water on a section lifter and transferred to a watch glass of the sodium or potassium hypochlorite solution. If the sections are made from material which has been preserved in alcohol, this solution should be used cold, but usually it must be warmed if it is to have the desired effect on materials which have been resurrected from a dried condition. In either case the operation should be watched very carefully under a low power of the microscope, and the action of the hypochlorite should be discontinued as soon as the cells are seen to be free of their contents. If the mounter is completely inexperienced in this field and is unable to determine the point at which the operation should be stopped, it is recommended that a single section be taken and the skeletonizing followed under a microscope while it is timed. It will be seen that when the operation has gone too far the finer cell walls present will be dissolved by the solution. If the period at which the first of the cell walls dissolves is carefully recorded and one half of this time taken for the subsequent sections, these will be cleaned perfectly without the slightest risk of damage to their walls. After they are removed from the hypochlorite solution, the sections should be washed thoroughly in several changes of distilled water and then passed into a solution of 1 per cent acetic acid. Here they are rinsed several times and then rewashed in ordinary water until the wash water no longer smells of the acetic acid. The skeletonized sections from as many roots as it is desired to cut at one time should be accumulated in water until one is ready to stain them, or they may be preserved indefinitely in 90 per cent alcohol.

The stain which is recommended in the present case is freshly prepared for use by mixing equal parts of a 0.2 per cent acid fuchsin solution and a 0.2 per cent iodine green solution. The mixed stains do not remain usable for much longer than one day, but the two separate stock solutions may be kept for an indefinite period. The differentiating solution, which is 1 per cent acetic acid in absolute alcohol containing 0.1 per cent iodine, is also stable. The staining solution should be placed in a small corked vial or stoppered bottle, and the sections transferred to it from the water. They should remain in stain for about 24 hours. It is recommended that they should not be left longer than 36 hours because they may suffer from a precipitate over the surface. For this reason it is desirable to accumulate as many sections as possible before starting the process. When the staining period is concluded, the contents of the vial should be tipped out into a large watch glass. It will usually be found that some of the sections remain behind, stuck to the side of the vial from which they are removed. Under no circumstances should the vial be rinsed with anything except the staining solution, which should be poured back from the watch glass (the sections will have settled to the bottom), swirled around, and returned to the watch glass. In this way in a short time all the sections may be removed from the vial to the watch glass. In a second watch glass or even a small crystallizing dish is placed an adequate quantity of the differentiating solution. Each section is removed individually with a section lifter from the stain and placed into the differentiating solution, where it may be watched under the low power of a microscope as the dish is rocked gently from side to side. Differentiation will usually take place within two or three minutes and may be determined without the least difficulty when the lignified tissues are found to be of a bright clear green, leaving a bright red in the nonlignified tissues. This process of differentiation is also one of dehydration, so that the sections may now be removed with a section lifter from the differentiating solution and placed in a clearing agent. The author's preference is for terpineol which has all the advantages of clove oil without the disadvantage of making the sections brittle so that they crack on mounting. All the sections may be passed through the differentiating solution and accumulated in terpineol. They may remain in terpineol until removed to a slide, where they are covered with balsam and mounted in the normal manner.

Sections prepared in this manner are permanent, and the process is so simple that it can be recommended most warmly as an introduction to plant section-staining techniques for an elementary class. The sections, however, are differentiated clearly enough to be used for instructing a class at any level, and generally they will be found much better for this purpose than the complex quadruple-stained sections in which the cytological detail all too often tends to obscure the clarity of morphological detail, the chief requirement in this type of teaching.

Summary

1. Cut hand sections of plant material hardened and preserved in alcohol. Accumulate sections in water.
2. Place sections in 1 per cent sodium or potassium hypochlorite until cell contents have been dissolved, leaving clean bleached skeleton.
3. Wash in water, rinse in 1 per cent acetic acid, and wash again in water.
4. Stain 24 hours in equal parts 0.2 per cent acid fuchsin and 0.2 per cent iodine green.
5. Differentiate sections individually in absolute alcohol containing 1 per cent acetic acid and 0.1 per cent iodine.
6. Transfer directly to terpineol and mount in balsam.

Example 10

Preparation of a Transverse Section of the Small Intestine of the Frog Stained with Celestin Blue B-eosin

This is the simplest example of paraffin sectioning which can be imagined and it may well serve as an introduction to this type of technique either for a class or for an individual. The intestine of a frog has been selected, rather than of any other animal, because of the availability of this form in laboratories, but any small animal may be substituted in its place.

Before killing the frog, it is necessary to have on hand a selected fixative. Since this is intended to be an example of the utmost simplicity, it is suggested that the cupric-nitric-paranitrophenol mixture of Petrunkewitsch (see p. 11) be employed. This fixative is entirely foolproof because objects may remain in it for weeks without damage, and it also permits excellent afterstaining by almost any known technique. If only a piece of intestine is to be fixed, 100 cc. of fixative will be sufficient. There is no reason why any other organ in the animal (with the exception of the central nervous system) should not be preserved in this fluid for subsequent investigation.

The frog is killed by any convenient method, but it is usually best for histological purposes to sever a large blood vessel and permit as much blood as possible to drain out from the heart before opening the abdominal cavity and removing the intestine. One or more lengths of about one third of an inch should be cut from the intestine and then transferred directly to a fixative where they may remain from a few hours to several weeks.

When they are next required, the specimens should be removed from fixative, washed in running water for a few hours, and then transferred directly to 70 per cent alcohol. The easiest method of washing objects of this size in running water is to take one of the coplin jars previously described, fill it with water and insert the specimen, and then attach a cover of coarse cheesecloth with a rubber band. The jar is placed in the sink and a narrow stream of water permitted to fall on it from the tap. It will be found that the specimen will swirl around in the jar in a most satisfactory manner. This simple device saves all the trouble of rigging up glass tubes and boring corks to make the cumbersome apparatus sometimes recommended for the purpose.

The actual procedure of embedding has already been described in some detail. The specimen is transferred after 24 hours in 70 per cent alcohol to 96 per cent alcohol. It is better to use a large volume of this alcohol and to suspend the object in it than it is to use relatively small volumes frequently changed. It is recommended, therefore, that a wide-mouthed stoppered jar of about

500 cc. capacity be obtained and a hook inserted into the center of its stopper, from which the object can be suspended. The majority of wide-mouthed, glass-stoppered jars have a hollowed undersurface which may be filled with plaster of Paris, and a glass hook, which is easily bent from thin glass rod, may be inserted in the liquid plaster. Naturally, this must be done some days beforehand, and the plaster must be thoroughly dried out in an oven before the jar is used for dehydrating. If the worker does not wish to go to this much trouble, it is also easy to screw a small metal pothook into the undersurface of a plastic screw cover for a jar of the same size. Alcohol is so hygroscopic, however, that it is better to employ a glass-stoppered jar, the stopper being greased with stopcock grease or petrolatum for a permanent setup. An object as coarse as the one under discussion may be suspended in a loop of thread or cotton directly from the hook, or, if this is not desirable, may be enclosed in a small fold of cheesecloth for suspension. After 24 hours in this volume of alcohol, the object will be completely penetrated by the 96 per cent alcohol and should be transferred to absolute alcohol, using the same volume in a jar of similar construction. It is useful to place about a quarter-inch layer of anhydrous copper sulphate at the bottom of the absolute alcohol jar, not only to make sure that the alcohol is absolute but also to indicate, as it changes to blue, when this jar should be removed from service. Of the many dealcoholizing (clearing) agents which may be used, the author would select benzene in the present case because it is less liable to harden the circular muscles of the intestine than is xylene. Since benzene is lighter than absolute alcohol, it is not possible to employ the hanging technique for clearing, and the object should be placed in about 25 cc. of benzene, which should be changed when diffusion currents no longer rise from the object. This is likely to be about six hours for an object of the size under discussion, and a second bath of at least six hours should be given.

It is necessary to select the medium in which embedding is to be done. The author would recommend the rubber paraffin of Hance (see p. 56) which must, of course, have been prepared some time before. The melting point of this medium is about 56° C. The oven should contain three stender dishes (see Fig. 5) as well as a 500 cc. beaker containing about a pound of the embedding medium. The object is removed from benzene, drained briefly on a piece of filter paper, and placed in one of the stender dishes which has been filled to the brim with the molten embedding medium. Under no circumstances should a lid be placed on the stender dish since it is desirable that as much as possible of the benzene should evaporate while the process of embedding is going on. After about three hours the specimen should be removed to fresh wax in the second stender dish, where it may remain about two hours, and then to the third stender dish, where it should not remain for more than one hour.

Shortly before the end of this last hour, a decision should be made as to what type of vessel is to be used for casting the block. It would be difficult to improve on a paper box (see p. 59) for this object. When the box is made (it should be of ample size), it is moistened at the bottom and placed on a slab of glass in the manner previously described (see p. 60). It should then be filled about halfway with embedding material from the beaker and allowed to remain until about half of this wax has congealed on the bottom. An object like the one under discussion is better handled with old forceps than with a pipette. The forceps should now be warmed in a flame to well above the melting point of the wax and moved backward and forward across the surface to melt the solid film which has formed. Then the object is picked up rapidly from its stender dish, placed in the wax, and enough fresh wax from the beaker added to make sure that there will be as much solid wax above as there is underneath the specimen. Blocks of this nature shrink greatly, and it will probably be best to fill the box entirely. As soon as the box has been filled, the forceps should be warmed again and passed backward and forward around the object to make sure that no film of unmolten wax, which would cause it to cut badly, remains. The wax in its box should now be blown on until it starts to congeal on the surface. Then it is picked up very carefully with the fingers and lowered into a dish of water at room temperature (the water does not quite reach the top of the box). If it is thrust under the surface at this point, all the molten wax will come out, and the block be rendered useless. As soon as the block has congealed throughout, it is thrust under the surface of the water and something is laid on it to keep it at the bottom. It should be left in the water for at least five or six hours or much better, overnight.

The block should be trimmed so that there is at least as much wax on each side of the object as there is in the object itself. This amount of wax would be excessive for serial sections, but for the preparation of individual sections of this type in an example given for the benefit of the beginner, this quantity is desirable. When the microtome is set up and the knife is sharpened in the manner previously described, the block is mounted. This process has been described in some detail (see p. 64) and need not be repeated here. The block having been trimmed to size and mounted, there remains only the actual cutting. The handle of the microtome should now be rotated rapidly and the beginnings of the sections observed. There is no need to worry if the section curls to one side or the other during this preliminary period since the entire thickness of the block will not be cut until 20 or 30 sections have been removed.

As soon as the knife is seen to be approaching the object and the block in its entirety is being cut, the ribbon must be observed most carefully to see that it is suffering from none of the common defects indicated in Table 1. Should the ribbon not be coming perfectly, various suggestions given in Table 1 may be tried until a perfect ribbon is secured. Since, in this case, a series of sections is not needed, it is unnecessary to cut a longer ribbon than one which will contain the actual number of sections required with a few left over for emergencies. However, it is a great mistake to throw away partially cut blocks of this nature since they may be stored in a glycerin-alcohol mixture quite indefinitely, and one never knows when further sections may be required. The block should be labeled before being placed in its solution. This is easily done by writing the appropriate label on a piece of paper and fusing this into an unwanted portion of the block with a hot needle.

Each individual section is now cut from the ribbon and mounted on the slide in the way described on page 67. Before actually using Mayer's albumen to mount the sections, it is necessary that the slides be cleaned. No two people have ever agreed as to what is the most desirable method of doing this. The author rubs the slide briskly with 1 per cent acetic acid in 70 per cent alcohol and dries it by waving it in the air. Another method of cleaning the slide, which yields equally good results, is given on page 49. After the sections are cut, several drops of the diluted adhesive are placed in the center of each slide. One of the individual sections is taken up with the tip of a moistened brush and placed on the adhesive. As soon as the section has been placed on the fluid, the slide is lifted up, warmed carefully over a spirit lamp until the section is flat but the paraffin not melted, and then the superfluous liquid removed carefully with the edge of a filter paper. One cannot in this instance, as though he were mounting ribbons, drain the fluid from the corner of the slide. The slide is put in a warm place to dry. If the drying period is to be prolonged, it is well to place a dust cover over the slide since grains of dust falling upon it will adhere just as tenaciously to the adhesive used as will the specimen itself.

It is proposed in the present example to stain the slide in the simplest possible manner with celestin blue B followed by eosin. The formulae for these stains will be found in Chapter 3. Of course, any of the other dyes recommended there may be substituted.

When the section is perfectly dry, it is turned upside down, and light is reflected from it to see whether or not the section is adherent to the glass. If there is any air gap between the section and the glass, a most brilliant mirror will be formed, so that, in a preparation as simple as this, the slide had better be thrown away. Those slides which are perfectly adherent are warmed over a flame until the wax is melted and then dropped into a jar of xylene, where they remain until a casual inspection shows the whole of the paraffin to have been removed. They are put into another jar of xylene for at least five minutes and then into a jar of equal parts xylene and alcohol for a further five minutes. This treatment is followed by five minutes in absolute alcohol and then by direct transference to distilled water. After the slides have been in distilled water for a few minutes, each slide should be lifted and inspected to make sure that the water is flowing uniformly over both the slide and section. If it tends to be repelled by the section or a meniscus is formed around the section, this is evidence that the wax has not been sufficiently removed, and the slide must be transferred first to 96 per cent alcohol to remove the excess water, then to absolute alcohol until perfectly dehydrated, and finally through absolute alcohol to xylene, where it remains until the wax has been completely removed before being brought down again as previously indicated. The slides may be taken down through xylene and alcohol one at a time and accumulated in distilled water until they are required. When all slides have been accumulated in distilled water, they are transferred to the celestin blue B staining solution. The time in this varies, but 10 to 15 minutes will probably be sufficient to stain the nuclei black. One of the most useful features of this stain is that it is almost impossible to overstain in it. It is also possible to leave sections overnight without staining the cytoplasm to a degree which requires differentiation. After the nuclei are black, therefore, or after a time convenient to the operator has elapsed, the sections are transferred to fresh distilled water where they are thoroughly washed. Then each slide is taken individually and dipped up and down in the eosin solution until a casual inspection shows the background to be yellowish pink. The staining of the background in a case like this is entirely a matter of choice; some people prefer a faint stain and others a darker stain, although it must be remembered in judging the color that the section will seem darker after it has been cleared than it does in water.

As soon as the time required to produce the desired degree of staining has been found by staining one slide, the remainder of the slides are placed in the eosin solution, left the appropriate time, and then transferred and left in distilled water until no more color comes away. The slides are passed from distilled water to 96 per cent alcohol where they are left for about five minutes. Then they are passed to fresh 96 per cent alcohol where they are left for five or six minutes before being passed to absolute alcohol. The purpose of using the 96 per cent alcohol is not to diminish diffusion currents but simply to save diluting the absolute alcohol by passing slides directly from water to it. After the slides have remained for five or six minutes in absolute alcohol, a single slide is passed into the absolute alcohol-xylene mixture for perhaps two minutes and then passed to xylene. On examination the slide should be transparent with only a faint opalescence. One of the commonest faults in mounting sections is to dehydrate them imperfectly. If there is any water, which has been carried through the process, in the xylene (in which water is soluble to the extent of about 0.2 per cent), this water will be extracted by the section which is in itself an excellent dehydrating agent. There is a world of difference between a perfectly cleared (that is, glass-clear) slide and one which is only more or less dehydrated so that it appears faintly cloudy. If the slide does not appear to be sufficiently dehydrated, all the remaining slides should be transferred to fresh absolute alcohol and another one tried. When it has become apparent from the examination of the test slide that dehydration is perfect, the remaining slides may be run up through absolute alcohol and xylene and accumulated in the final jar of xylene.

Each section must be mounted in a solution of dried balsam in xylene. All that is required for mounting is the cleaning of the appropriate number of coverslips for which, in the present instance, a ¾-in. circle would be admirable. Again, individual preference may be consulted in the manner of cleaning. The author cleans his coverslips in the same manner as he cleans his slides by wiping them with a weakly acid alcohol solution. Each slide is taken individually, drained by its corner, and laid on a flat surface; a drop of mounting medium is placed on its top. Then the coverslip is placed on the mounting medium and pressed down with a needle. It should not be pressed absolutely into contact with the slide, or too thin a layer of mounting medium will be left. Some experience is required to judge when the coverslip has been pushed down far enough. If this is done skillfully, the surplus mounting medium will form a neat ring around the outer surface of the cover. If it does not do so, care, at least, should be taken that no portion of the cover is devoid of surplus mountant which will be sucked under the coverslip as the solvent evaporates. These slides should be left to dry at room temperature for about one day and then placed on a warm plate for about a week. After they are dried, the surplus dry mounting medium should be scraped off with a knife, and the excess, remaining after scraping, removed carefully with a rag moistened in 96 per cent alcohol.

Summary

1. Bleed an anesthetized frog to death. Fix short lengths of intestine in Petrunkewitsch's fluid for at least 1 day.

2. Wash specimen in running water for some hours. Dehydrate, clear, and embed in wax.
3. Trim block and attach to microtome. Cut ribbon of sections and mount single sections or short lengths on clean slides, using diluted Mayer's albumen to flatten sections. Dry thoroughly.
4. Heat dried slides until wax melts. Place in xylene until all wax is removed. Remove xylene with absolute alcohol and transfer slides to water.
5. Transfer to celestin blue B solution until nuclei are stained black (10 to 15 minutes). Wash in water.
6. Transfer to 1 per cent eosin until sections appear stained sufficiently. Wash in water.
7. Dehydrate, clear, and mount in balsam.

Example 11

Preparation of a Transverse Section of a Stem of Aristolochia Stained by the Method of Johansen

In the preparation of a transverse section of a root described in Example 9, nothing was required except that an outline of the cells be shown as a skeleton and that this skeleton be differentiated as to woody and nonwoody tissues.

The present example describes the preparation of a section of a plant stem preserved, sectioned, and stained in such a manner as to differentiate all of the components present. The technique to be described could be applied equally well to a leaf or any other part of a plant; the stem of an Aristolochia has been selected only because of its wide use in teaching.

First it is necessary to collect and fix the material. The choice of fixative is not of any great importance, but the majority of workers prefer a "Craf" fixative, such as the formula of Lavdowsky given on page 8, for this type of material. About a pint of this solution should be prepared immediately before it is required for use.

Great care must be taken in the selection of a stem. If a second- or third-year growth is taken, it will be too woody to cut satisfactorily, while a new growth of early spring will not be differentiated sufficiently to justify applying a complex stain of the type used in this example. The best Aristolochia material is usually obtained from the new season's growth when it has partially ripened toward the end of August. Care must be taken that the piece of stem is not so cut as to permit the introduction of air into the transporting vessels since this will greatly increase the difficulties of subsequent manipulation. Unless the stem can be cut in the early morning while it is completely turgid, it is best to pull down a branch of the vine and make the first cut under the surface of water in a bucket. About a foot of stem is pushed under the water and cut off. The piece should be transferred immediately to a large volume of fixative and stored in the dark for about three days. At the end of this time the central portion of the stem should be cut into one-half inch lengths with care so as to avoid crushing, and the pieces washed overnight in running water.

The process of dehydrating, clearing, and embedding plant material is exactly the same as that used for animal material, so that the directions given on the handling of frog intestine in Example 10 may be used without modification. Sections should be cut from the block at about 10 microns thickness. These, if desired, may have the wax dissolved from them directly and be handled as were the sections of root in Example 9. It is much more convenient, however, to mount individual sections or short lengths of ribbon on the slide in the manner described in Example 10. While these slides are drying, it is well to make up the solutions necessary for Johansen's technique.

It will be noticed by reference to page 28 that these require both methyl cellosolve and tertiary-butyl alcohol. Neither of these are common laboratory reagents, and their availability should be checked before commencing the technique. There will be required four staining solutions (safranin, methyl violet 2B, fast green FCF, and orange G), three differentiating solutions (which consist of mixtures of various solvents in proportions which must be followed closely), and one special dehydrating solution which must be used to avoid removing the stain in the final dehydration.

The worker thus should have in front of him the following coplin jars of reagents arranged in two rows. The jars in the front row should contain xylene, absolute alcohol, 95 per cent alcohol, and 70 per cent alcohol. Those in the back row should contain, in order, the safranin solution, the methyl violet solution, the first differentiating solution, the fast green FCF, the second differentiating solution, the orange G, the third differentiating solution, and the special dehydrating reagent. A large dish should be available in the sink, in which the slides can be washed in running water.

The description to follow will be based on the handling of a single slide, though there is nothing to prevent racks of slides from being taken through as readily as a single example.

A slide is heated over a flame until the wax is melted. Then it is placed in xylene until the whole of the wax has been dissolved. It is transferred to absolute alcohol for the removal of the xylene, to 95 per cent alcohol for the removal of the xylene-contaminated absolute alcohol, and then to 70 per cent alcohol. The slide is placed in safranin and left from 1 to 3 days or until the nuclei are seen to be deeply stained in red. Then the slide is washed in running water until no more color comes away. This initial staining in safranin is the point at which the technique is usually interrupted since the slides may be left in the staining solution indefinitely without damage, while the subsequent operations must be conducted consecutively.

The thoroughly washed slides are transferred to the methyl violet solution for 10 to 15 minutes. On removal, they should be rinsed briefly in running water to remove the surface stain before being differentiated in the first differentiating solution from 10 to 15 seconds. Then they are placed directly into the green staining solution where they remain from 10 to 20 minutes or until their red-purple color is changed to a greenish hue. The slide is taken from the green solution, drained from the corner, and the back of it is wiped. A very brief rinse (5 to 10 seconds) in the third differentiating solution precedes the transfer to the clove oil-orange G solution. It is perfectly satisfactory to have this solution available in a drop bottle from which it can be poured on the slide, although, if any large number of sections are to be handled, a coplin jar is usually more convenient.

The slide should be removed from the orange stain at intervals and examined under a microscope. Staining should be discontinued when the cytoplasm of the cell changes from a muddy grey color to a clear bright orange. This will normally take from 3 to 5 minutes, although occasionally, a longer period of time is required. As soon as the required color has been reached, the slide is dipped up and down three or four times in the special dehydrating agent, the primary purpose of which is to remove the excess orange from the surface of the slide. Then the slide is transferred to xylene, in which the special dehydrating agent should be removed completely, before being mounted in balsam in the ordinary manner.

Summary

1. Select a suitable Aristolochia stem and fix in Lavdowsky's fluid.
2. Wash short lengths of stem in running water, dehydrate, clear, and embed in wax.
3. Mount individual sections or short lengths of ribbon on slides, dry, dewax, and transfer to 70 per cent alcohol through the usual reagents.
4. Stain in Johansen's safranin from 1 to 5 days.
5. Wash thoroughly in running water and stain in 1 per cent methyl violet 2B for 10 to 15 minutes.
6. Rinse in water and differentiate 10 to 15 seconds in the first differentiating solution.
7. Stain in fast green FCF from 10 to 20 minutes.
8. Rinse in third differentiating solution 5 to 10 seconds and stain in orange G 3 to 5 minutes.
9. Rinse off orange G in special dehydrating mixture, remove this mixture with xylene, and mount in balsam.

Note: The formulae for staining, differentiating, and dehydrating solutions used above are given on page 28.

Example 12

Demonstration of Spermatogenesis in the Rat Testis, Using the Iron-Hematoxylin Stain of Heidenhain

The laboratory white rat is one of the best forms in which to show spermatogenesis for the reason that it has a continuous breeding period, and all stages are available, therefore, in almost every section examined.

The rat selected should be a young male. It is killed most conveniently with chloroform. The scrotal sac is opened by a median incision, and the testes are removed, trimming away the epididymis. The testes should be placed on a clean glass plate and slashed with a sharp scalpel or razor about two thirds of the way through by cuts spaced a few millimeters apart before being thrown into the fixative solution selected. Few fixatives for this purpose can surpass Allen's fluid (see p. 11). At least 100 cc. of fixative should be employed for a normal-sized testis, and the bottle containing it should be reversed once or twice during the first few hours to avoid the accumulation of diluted fluid at the bottom. The time of fixation is not of any great importance but, in any case, should be overnight and, in general, should not exceed a few weeks. After the object is removed from the fixative, it may be washed for about an hour in running water before being transferred to 70 per cent alcohol to complete the removal of the picric acid. It must be emphasized that washing a specimen in water, after picric acid fixatives, results in a great deal of vacuolation of the cytoplasm. Of course in the present instance, this does not interfere with the object being studied. After three or four changes of 70 per cent alcohol—the testes remain in a large volume of solution for at least two or three days between changes—the final removal of the picric acid may be accomplished by adding a small quantity of dry lithium carbonate to the alcohol used for washing. It will be impossible to remove all the yellow color, some of which is occasioned by the combination of the picric acid with the albuminoids present, but the last alcohol used for washing should be only very faintly tinted with yellow. It is not the color of the fixed material to which objection is raised during the passage of the material through paraffin; it is the fact that, unless most of the picric acid is removed, there will be crystallization with consequent damage to the tissues.

Small pieces may now be removed for embedding from the testis itself. It is best to select pieces about a millimeter in from the surface and of about a 2 mm. side. These should be embedded in paraffin and cut about 5 microns thick in the usual manner. Then the sections should be attached to slides, particular attention being paid to the fact that the slides are clean and that not too much

Mayer's albumen, which may interfere with differentiation or staining, is used.

The dry sections attached to the slides should be warmed on the underside until the paraffin melts, placed in xylene until the paraffin is removed completely, and then run down in the ordinary way through absolute alcohol and lower percentage alcohols to distilled water. Then they should be lifted from the distilled water and examined carefully. If there is a tendency for the water to gather in droplets on the slide or, if upon shaking the water from the slide, each section appears to retain an adherent coat of water around itself, it is an indication that either the wax was not removed properly in the xylene or that the xylene itself is so old as to have a wax content too high to be useful. Such slides must be returned through the alcohols to absolute alcohol and thence to clean xylene where they should be left for a few minutes before again being brought down to the water and reexamined. There is no more common cause of the failure of the stains to take than the imperfect removal of the wax.

Only two solutions are required for staining. These are a 2.5 per cent solution of ferric alum and a 0.5 per cent solution of hematoxylin. The only difficulty in making the ferric alum solution is to secure a pure and unoxidized sample of the reagent. Most of the crystals in a new bottle are of a clear violet color, but after a bottle has been opened for some time, particularly if the stopper is loose, most of the crystals become covered with a brownish deposit which must be scraped off with a knife before the solution is prepared for staining. If the brown powder on the outside of the crystal forms a layer of any thickness, it is best to reject the whole and secure a fresh supply of the reagent. Hematoxylin itself has little staining effect; the color is produced by the formation of lakes with hematein, an oxidation product of the hematoxylin. It was customary in former times to prepare large quantities of solution, which were kept with the stopper loose in the bottle for a period of at least one month before use. For the purpose of Heidenhain's technique, however, it is far more important that a small quantity of the ferric alum should be carried over into the hematoxylin solution than that the latter should be aged. The staining will be found both simpler and more effective if a few drops of hematoxylin are placed in the iron alum solution and a few drops of the iron alum solution are placed in the hematoxylin. Both solutions, of course, should be filtered immediately before use if the finest slides are required because chromosome figures in a rat can be obscured by a very small particle of dust.

Next the slides are taken from distilled water and placed in the mordant solution. It matters little how long they remain in this solution though the usual directions call for keeping them there overnight. This varies with every type of tissue studied and is greatly dependent on temperature. If the solutions are heated to 50° C., with the understanding that this will cause a swelling of the section and a general obscuring of the finer details, the period may be shortened to as little as 10 minutes. The finest stains, however, are those secured by leaving the sections in the mordant solution at room temperatures overnight. On removal from the mordant solution, the sections should be rinsed *very* briefly in distilled water; the purpose of the rinse is to remove the surplus mordant from the surface of the slide without extracting it from the tissues. Then the slides are placed in the hematoxylin solution where they should remain for approximately the same period as they have been in the mordant; the length of time is not important, though from 3 to 24 hours is the customary period. Sections may be removed from time to time from the staining solution and examined with the naked eye. Successful preparations show the sections to have been blackened completely, although a slight bluish tinge in the black is permissible. If they have not become blackened completely in 24 hours, it is necessary only to place them, after a brief rinse, in the mordant solution and leave them there for another 24 hours before returning them to the stain.

If, however, the sections are sufficiently blackened on removal from the staining solution, they may be differentiated; that is, the hematoxylin stain extracted from all portions of the sections except the chromosomes, which are to be studied. This is done customarily with the same solution in which they were mordanted, though, of course, a fresh solution or a stronger solution may be employed if desired. Differentiation at the commencement of the process goes relatively slowly, so that all the slides, which are carried in a glass rack, may be removed and placed in the 2.5 per cent iron alum without any very great care. The actual time in which differentiation takes place cannot be forecast since it depends on a large number of uncontrollable factors; it is never less than five minutes nor is it very often more than a few hours. Sections, therefore, should be withdrawn from the iron alum every four or five minutes and examined briefly under the low power of a microscope. It is a matter of great convenience for the controlling of differentiation of chromosomes in this type of preparation if an ordinary student microscope (of the type customarily used in laboratory) can be lifted with a glass plate over the stage, so that one can, without fear of damage to the instrument, place a slide, wet with iron alum, on the surface of the stage for examination. No more common accident takes place than the placing of the slide upside down on the surface of the stage with the subsequent loss of all the sections. This will be avoided readily if the worker will make it a matter of routine; as he lifts the slide from the mordant, to hold it at an angle between himself and a light source so that the light is reflected from the surface. If the sections are, as they should be, on the upper surface of the slide when it is so placed, they will appear to be double through the reflection from the undersurface as well as the uppersurface of the glass. A good rule is never to place a slide which has just been taken from a fluid on the stage of the microscope until one has seen the double reflection.

If a low-power examination of the section shows the nuclei to be standing out clearly, the entire tray should be removed to distilled water because from this time on differentiation is very rapid, and each slide must be controlled separately. If, however, the nuclei are not sharply defined and a considerable degree of black or bluish color remains in the background, then the entire tray may be left in the iron alum for as long as is necessary. When this preliminary differentiation down to the distinction of the nuclei under low-power examination has been completed, it is necessary to continue differentiation while examining the slides at frequent intervals under a very high power of the microscope. It is convenient to have available a water-immersion objective. It is obviously impossible to place immersion oil on a wet slide, while short working distance of a high-dry objective renders it particularly liable to cloud up from the evaporation and recondensation of the water. Water-immersion objectives are usually of 3 mm. equivalent focus, and this gives a sufficiently wide field to permit differentiation to be observed, while at the same time it has sufficiently high magnification for satisfactory control. Each slide should be taken separately, returned to the 2.5 per cent iron alum for a few minutes, and then reexamined. The various phases of mitosis and meiosis do not retain the stain to the same degree, and care must be taken that the color is not washed completely out of the other chromosomes as a result of examination only of metaphase figures in which the color is retained longer than in any other. A great deal of practice is required to gauge accurately the exact moment at which to cease differentiation, which may be stopped almost instantly by placing the slides in a slightly alkaline solution. In Europe most tap waters are sufficiently alkaline for the purpose and are generally specified, but in the cities of the United States it is often best to add a very small quantity of lithium carbonate or sodium bicarbonate to the water used to stop differentiation. Slides may be left in this for any reasonable period of time; the process is complete when the slide turns from a brown to a blue color.

Then the slides are rinsed in distilled water, graded up through the various percentages of alcohols, dehydrated, cleared, and mounted in balsam in the usual manner. A section on a slide, which is required for examination over a long period of time, should be some distance from the edge of the coverslip, for, as the balsam oxidizes inward from the edge, it tends to remove the color of the stain from the chromosomes, leaving them a rather unpleasant shade of brown. If this happens to a valuable slide, however, the matter can be remedied by the utilization of a green light which will make the chromosomes appear black again.

Summary

1. Remove the testes from the scrotal sac of a young male rat and trim away the epididymis.
2. Slash the testes deeply with a razor, making cuts two or three millimeters apart. Fix in at least 100 ml. of Allen's fluid from 1 to 7 days.
3. Wash for about an hour in running water before removing small pieces to 70 per cent alcohol. Change alcohol daily until no further color comes away.
4. Dehydrate, clear, embed in wax, and cut 5-μ sections. Attach sections to slide. Dewax dried slides and grade down to water through usual reagents.
5. Mordant overnight in 2.5 per cent iron alum.
6. Rinse briefly and stain overnight in 0.5 per cent hematoxylin.
7. Rinse briefly and place in 2.5 per cent iron alum until chromosomes are clearly differentiated.
8. Wash in alkaline tap water until sections are blue. Dehydrate, clear, and mount in the usual manner.

Example 13

Preparation of a Transverse Section of the Tongue of a Rat, Using Celestin Blue B Followed by Picro-acid-fuchsin

The chief difficulty in preparing a transverse section of the tongue is to avoid the hardening of the muscle, which tends to become brittle either if imperfectly fixed or handled with undesirable reagents in any stage of the proceedings. Therefore, it is recommended that the following description be followed rather closely, for it can be adapted almost without variation to any other heavily muscularized tissues, which it is desired to stain.

The tongue may be removed most easily by severing the articulation of the lower jaw, and removing this together with the adherent tongue, which may be detached with a short scalpel or cartilage knife. A portion of the tongue, approximately 5 mm. in length, is cut off and placed in a large volume of the selected fixative.

Though opinions vary widely as to the most desirable fixative to employ for muscularized tissues, it may be said at once that no alcoholic solution or solution containing picric acid or mercuric chloride can be employed under any circumstances. The author's choice would be the solution of Petrunkewitsch, which he has employed most successfully on a variety of heavily muscularized tissues. This formula would also provide an excellent premordanting for the staining techniques which follow. Whatever formula is selected, however, a very large volume should be employed and permitted to act for no longer than is necessary to secure the complete impregnation of the tissues. If the operator does not wish to employ a copper formula, it may be suggested that he use any formula containing nitric acid or one of the weaker dichromate mixtures. When the piece has been fixed successfully, it must be washed overnight in running water and then dehydrated. The process of dehydrating, clearing, and embedding is the point at which most muscularized tissues become unmanageable. Nothing, of course, can counteract the effect of improper fixation, but even with good fixation the utmost attention must be paid to the selection of dehydrating agent, clearing agent, and to the temperature at which the embedding takes place. It has been the experience of the author that the newer substitutes for alcohol in dehydrating tend to harden or render brittle muscular tissue to a greater extent than does the more old-fashioned method of using ethyl alcohol. There is little choice in the matter of clearing prior to embedding, for it has been found by numerous workers that benzene has less hardening effect on muscular tissue than has any other agent.

Unless very thin sections are to be cut, it is recommended that a wax of no higher melting point than 52° C. be employed and that embedding be conducted exactly at this temperature. It is far easier to do this by the method of an overhead heating light placed above a tube of paraffin than by thermostatic control. It may be stated categorically that should the temperature be permitted to rise above 56° C. it would be better to throw the preparation away than to waste time endeavoring to section it. Paraffin sections are cut from the block by the standard method, stranded, and caused to adhere to the slide by egg albumen. These sections, however, may be lost because muscularized tissues tend to absorb water very readily when in paraffin section, so that, if the sections are flattened for a prolonged period in contact with large volumes of warm water, they will tend to expand more than the surrounding wax with the result that they will arch away from the glass support and inevitably become detached while being deparaffinized. Therefore, it is recommended that, as soon as the sections are flattened, they should be pressed to the slide with a piece of wet filter paper, rolled into position with a rubber roller, and dried with the maximum possible speed.

As soon as they are dried, the sections are deparaffinized by the usual techniques and taken down to distilled water where they may remain until one is ready to stain them. Celestin blue B is selected as the nuclear stain in this instance because the contrast of muscularized tissues is far better brought out with the aid of a picro-contrast than by any other method. These picro-contrasts are, however, so acid that hematoxylin-stained nuclei are often decolorized in the course of counterstaining. The formula for the nuclear stain is given on page 21. The solution presents no difficulties of preparation and need not be rejected if it shows a slight precipitate at the bottom. The sections are passed directly into it from the distilled water and allowed to remain until an examination under the low power of the microscope shows the nuclei to be stained clearly and deeply. It is very difficult to overstain in this solution; though the time specified in the formula given is from 5 minutes to 1 hour, no damage will be occasioned should the sections remain overnight in the staining solution. After they are removed from the staining solution, they are rinsed in distilled water and accumulated in a jar of either distilled or tap water until it is time to counterstain them.

The solution of van Gieson (see p. 23) should be used for counterstaining. This stain requires little or no differentiation, so that the sections may be placed in it and examined from time to time until the muscles are seen to be stained a very bright red against a connective-tissue background of yellow. If a small quantity of red is picked up by the connective tissues, it will be removed in the process of differentiation. The time is not critical, but that given in the formula cited (2 to 10 minutes) will be found to cover the range normally necessary.

A slight difficulty will be occasioned in dehydration through the tendency of the picric acid to leave the tissues in the various alcohols employed. Either this may be prevented by dehydrating in a series of 1 per cent solutions of picric acid in the various alcohols, or it may be ignored completely according to the depth of yellow color which is to be retained. If on the contrary, a very pale yellow is desired, the sections may have to be put in 96 per cent alcohol, for a period of time beyond that necessary for dehydration, to remove the unwanted picric acid. Then the sections are cleared in xylene in the normal manner and mounted in balsam.

Summary

1. Place a 5-mm. slice of tongue in 100 ml. of Petrunkewitsch's fluid for 1 to 2 days.
2. Wash overnight in running water. Dehydrate, clear, embed in low melting-point wax, and cut 10 to 12-μ sections.
3. Flatten sections and squeeze to slide (see p. 69). Dry as rapidly as possible. Dewax dried sections and grade down to water through usual reagents.
4. Stain in celestin blue B until nuclei are deeply stained (5 minutes to 1 hour). Overstaining is not possible.
5. Rinse in water and stain in van Gieson's picro-acid-fuchsin from 2 to 10 minutes.
6. Dehydrate and differentiate in 95 per cent alcohol. Clear and mount in the usual way.

Example 14

Preparation of a Transverse Section of Amphioxus, Using the Acid Fuchsin-aniline Blue-orange G Stain of Mallory

Amphioxus is a difficult subject from which to prepare satisfactory sections; the more so as it is almost impossible nowadays to secure supplies of living Amphioxus and to fix them oneself, or to prevent the supplier from whom one secures the fixed material from using Bouin's fluid. If it is possible to secure the living animals, they should be fixed by the methods recommended for heavily muscularized material in Example 13. If this is not done, it is almost impossible to secure unbroken sheets of muscle in the transverse section unless one is prepared to sacrifice a certain amount of histological detail in the interest of morphological demonstration. It is also greatly to be regretted that popular demand has forced the biological supply houses to sell only the very large specimens, with the result that the sections are too large to be viewed at one time in even the lowest power commonly available on a student microscope. If any selection can be exercised, care should be taken to pick a specimen of not more than 2.5 mm. thickness, in order that it may be seen as a whole.

If, however, one is forced to use a Bouin-fixed specimen, it may be sectioned without too much difficulty, provided that it first is soaked overnight in 1 per cent nitric acid. This treatment, naturally, destroys much of the fine cytological detail and should not be applied to any specimen in which it is desired to demonstrate, for example, the detailed structure of the endostyle. The author, however, is always prepared to sacrifice such detail as this in a section desired for class demonstration to avoid having to answer endless questions as to what is this and that cavity which will be seen in a section of Bouin-fixed Amphioxus handled by routine methods.

Apart from this question of fracturing of the muscular layers, no difficulty will present itself in sectioning, and as many as 10-μ sections as are required should be accumulated. If it is desired to place on the same slide a collection of sections from different regions of the animal, reference may be made to the description of this procedure on page 131.

When the sections have been mounted on slides, deparaffinized, and graded down to water, it is recommended that they be treated overnight in a saturated solution of mercuric chloride and then washed in running water for at least six hours. This process improves the vividness of Mallory's stain almost beyond belief when it is applied to a section of Bouin-fixed material. The solutions used for Mallory's stain (see p. 25) present no difficulty in their preparation, though it is recommended that 1 per cent phosphotungstic acid be substituted for the 1 per cent phosphomolybdic acid specified in the original method. The sections, when they have been washed thoroughly after the mordanting in mercuric chloride, are placed in the 1 per cent solution of acid fuchsin for a period of about two minutes. This time is not critical; it is necessary only to make sure that the entire section is thoroughly stained. On removal, the sections are rinsed rapidly in water for the purpose of removing the surplus stain, and then are placed in the 1 per cent phosphotungstic acid until such time as the red stain has been removed entirely from the connective tissues. This may be judged partly by the cessation of the color clouds which rise from the section and partly by an examination under the lower power of the microscope to make sure that the septa between the myotomes are free from color. The specified time of 2 minutes is usually sufficient, and the sections will not be damaged however long they may be left. On removal from this solution, they are rinsed quickly in water and placed in the acid-methyl blue-orange G solution where they should remain for at least 15 minutes. The mistake is often made of leaving them for too short a time in this stain, for they will have the appearance of being deeply stained after an immersion of only a few moments. It does not matter how long they remain; it is the author's experience that soaking for at least 15 minutes discourages the subsequent removal of the blue from the tissues. After they are removed from this rather thick staining mixture, the slides are washed thoroughly in water. This wash is designed to remove not only the whole of the adherent stain from the slide, but also to permit the oxalic acid to be leached out of the tissues. No "differentiation" of the stain should take place in water, since the necessary differentiation is produced by the absolute alcohol used for dehydration in the next stage. It is quite impossible to take sections stained by this method up through the common graded series of alcohols, but no grave damage will be occasioned by the omission of this step. If, however, the operator is one who insists that his sections pass through a graded series, mixtures of acetone and water should be substituted for alcohol and water. When the sections reach absolute alcohol, they should be watched very closely, while being moved continuously up and down in the alcohol. The blue color will leave them in great clouds; these clouds will taper off quite rapidly, leaving a terminal point at which no color leaves for a moment or two before a slow stream again starts to appear in the alcohol. As soon as the initial color clouds cease, the sections should be removed to xylene, which instantly stops the differentiation. If the operator is uncertain of this method or is trying it for the first time, it is recommended that the slides be washed thoroughly in absolute alcohol but removed to xylene before the color clouds have ceased to leave the sections. Examination under the low power of the microscope will show these preparations to have dull purple muscle tissue and intensely blue connective tissue. A few trial sections should now be returned to absolute alcohol for a few moments, put back into xylene, and then reëxamined. It is possible by this means to exercise the most perfect control over the differentiation, which should be stopped when the muscles and nuclei are clear red and the connective tissues are a clear light blue. No attention should be paid, while differentiating, to any of the structures, such as the gonad, which by this method acquire a violet coloration which cannot be judged; the process should be controlled only by apparent contrast between the pure blues and pure reds in the section.

The stains used in this preparation are alkali sensitive, and it is a customary procedure in Europe to mount them in as acid a medium as possible. If one is using one of the synthetic resins, which are neutral, it is strongly recommended that the coverslip, before it is applied to the resin, should be dipped briefly in a strong solution of salicylic acid in xylene. This salicylic acid will dissolve in the resin and provide a permanently acid medium; with this precaution, the sections are permanent.

Summary

1. Fix a small adult Amphioxus in any good fixative. If only a Bouin-fixed specimen is available, soak it overnight in 1 per cent nitric acid.
2. Cut 10-μ paraffin sections in the usual way, attach to slide, and grade to water.
3. If fixative does not contain mercuric chloride, soak sections overnight in a saturated solution of this salt and wash for some hours in running water.
4. Stain in 1 per cent acid fuchsin for about 2 minutes.
5. Rinse quickly in distilled water and transfer to 1 per cent phosphomolybdic acid until no more color comes away (about 2 minutes).
6. Rinse quickly and place in Mallory's oxalic acid-methyl blue-orange G mixture for 15 minutes.
7. Wash thoroughly in water. Rinse in 95 per cent alcohol to remove adherent water. Differentiate each slide individually in absolute alcohol. Stop differentiation with xylene and mount in balsam.

Example 15

Preparation of a Series of Demonstration Slides, Each Having Six Typical Transverse Sections of a 72-hour Chick Embryo, Using the Acid Alum Hematoxylin Stain of Ehrlich

Example 3 described in some detail the manner in which a chick embryo may be removed from the yolk and fixed in a syracuse watch glass, where it is stretched by a collar of filter paper during fixation. Exactly the same procedure should be followed in the present instance, save that it is not necessary to leave the hole in the paper of a size larger than will accommodate the embryo itself. The same fixative recommended in Example 3 should be employed, and, after the removal of the fixative, the embryo should be embedded in paraffin by the technique described on page 57. Then the complete series of serial sections should be taken throughout the whole embryo, and the ribbons should be accumulated on a sheet of black paper in front of the worker.

It is presumed for the purpose of this example that the reader desires to prepare a series of slides for class use on each of which there will be arranged, in order, transverse sections through the regions of the eye, the ear, and the heart, and the anterior, middle, and posterior abdominal regions. In these regions will be found all that is required for the purpose of teaching an elementary class the development of the eye, ear, and heart, and the closure of the amnion and neural folds. It is necessary first to identify those sections which will show the required structure and isolate the portions of ribbon containing them. Provided that the sections are placed against a background of black paper, this is relatively simple with the aid of a long-arm, binocular, dissecting microscope, which may be swung over the ribbons and which will supply sufficient magnification to enable the regions of the ribbon to be identified by a competent microtomist. If the operator has had little practice at this, it might be desirable to stain the embryo in Mayer's carmine before embedding. Then each portion which contains the selected sections is cut from the ribbon with a sharp scalpel which is moved with a rocking motion, picked up on a camel's-hair brush, and transferred to another sheet of black paper. The remains of the ribbon may be thrown away.

The sections in each of the selected strips of ribbon are counted to determine the maximum number of slides which may be made—the ear sections are usually the limiting factor—and the pieces of ribbon trimmed, each to contain approximately the same number of sections. Then the required number of slides are cleaned and a few drops of the usual adhesive added to 25 ml. of filtered distilled water in a small flask.

The only difficulty of the procedure consists in persuading each section to occupy its correct place on the slide. This is carried out most easily by the following technique. A single slide is taken, placed in front of the operator, and covered lightly with the diluted adhesive. The fluid should extend to the edge of the slide but should not be raised in a meniscus sufficiently high to cause any appreciable slope of the fluid from the center of the slide toward the edges. Using a sharp scalpel, the end section is cut, with a rocking motion, from each of the ribbons. These sections are placed in the correct order but without any regard to symmetry on the surface of the fluid on the slide. To secure these sections in the required position, it is necessary to have two fine brushes, a mounted needle, and a bunsen burner or spirit lamp.

The last section (that is, that section which is to lie farthest from the label on the slide) is now secured in position with a brush held in the left hand, while the second section is maneuvered with a brush held in the right hand until its edges touch those of the first section. Both sections will be held together by capillary attraction when the brush is removed. The needle is warmed in the flame and used to fuse the edges of the sections together in two spots. If the entire edge is melted, it will cause a ridge which will prevent the compound ribbon from lying flat against the slide; two minute spots fused together with the point of the needle are sufficient to hold the section in place. The needle is laid down, and the brush again picked up with the right hand and used to guide the next section into its appropriate place. This section is spotted into position with the tip of the warm needle, and so on, until all the sections have been fused into a continuous ribbon. Then the slide is placed in the usual way upon the warm table until such time as the ribbons have flattened; they are drained in the manner described under the section on section cutting technique and then pressed to the slide with a wet filter paper and rubber roller, if this is the method of operation preferred by the technician. The compound ribbon, of course, may be guided into the center of the slide, while the latter is still wet, before it is pressed or dried. The sections are left, in the ordinary course of events, on the warm table until they are entirely dry before being dewaxed in xylene and brought down to 90 per cent alcohol through absolute alcohol in the usual manner.

Ehrlich's acid alum hematoxylin has been selected for this typical example because it is one of the best, though at the same time one of the most frequently misused, of the hematoxylin stains. The method given for its preparation should be rigorously followed; that is, the hematoxylin should be dissolved in a mixture of acetic acid and absolute alcohol, and then the glycerin, water, and ammonium alum should be added to the bottle, which should be shaken vigorously and allowed to ripen with the stopper loose for some months. "Artificially" ripened hematoxylin does not give as good a preparation, but there is no reason that this stain should not be prepared in half-gallon lots at routine intervals, so that a sufficiently ripened solution is always available. When it has once been ripened, which can be told both by its "fruity" smell and dark color, it remains in a fit condition to use for many years. One of the most frequently omitted precautions is the maintenance of the concentration of the ammonium alum by the addition of about 100 Gm. per liter to the bottle after it has been sufficiently ripened. This stain should never be diluted but should always be used full strength by the method now to be given.

All the slides, gathered together in a glass tray, (each slide may be treated individually) are taken from the 96 per cent alcohol and placed in the full strength Ehrlich's hematoxylin solution for a few minutes. The exact time is not important, but they should be examined at intervals to make sure that they are not becoming overstained. In the absence of experience, it is recommended that a period of 1 minute be used and that then they be examined under a lower power of the microscope, at which time the nuclei should appear quite densely stained, the background being only lightly stained. Each slide is removed individually from the tray, wiped on the underside with a clean cloth, and then "differentiated" by dropping 96 per cent alcohol (*never* acid alcohol) on it with a drop bottle or a pipette. It will be observed at once that the drops of the very thick hematoxylin solution are rolled back from the section as the 96 per cent alcohol drops on it and that, after a short time, the nuclei become more distinct and the background less distinct. The exact point at which differentiation should cease is at the will of the operator, but it is better, in general, since the sections are not to be counterstained, to discontinue differentiation when the nuclei are clearly defined against the background. Each slide is transferred directly to a saturated solution of lithium chloride in 70 per cent alcohol where it passes from a pinkish color to a deep blue. If the conventional method of differentiating these stains with acid alcohol is followed, it results in a hopelessly diffuse stain. The purpose of the 96 per cent alcohol is to utilize the surface tension of the stain to hold it in the nuclei, and, if the slide is placed in acid-70 per cent alcohol, it will be found that the stain diffuses out from the nuclei which, instead of appearing clear and sharp, appear, as it were, blurred around the edges as in an out-of-focus photograph. Differentiation by rolling back the stain with 96 per cent alcohol gives a clear, sharp stain which is as well differentiated as any of the iron alum mordant stains, but which has the advantages of giving a greater transparency and also staining the background sufficiently to render it apparent for class demonstration purposes.

The slides may remain in the saturated solution of lithium chloride in 70 per cent alcohol for as long as is required, being subsequently passed directly through the higher alcohols to xylene and mounted in balsam or some synthetic substitute for it.

This technique may, of course, be applied to accumulating and staining selected sections from any series cut from any material.

Summary

1. Remove a 72-hour chick embryo from the egg and fix it as described in Example 3.
2. Wash, dehydrate, clear, remove from paper support, embed in wax, and cut a complete series of 10-μ sections throughout the whole embryo.
3. Remove from the ribbon those portions from which sections are to be mounted.
4. Flood a clean slide with diluted Mayer's albumen. Place selected sections in order, but without regard to symmetry, on fluid.
5. Using two brushes, bring the two end sections into position. Fuse the edges together in two places with a hot needle. Join the other sections in order until a ribbon has been formed. Warm to flatten sections; drain and dry.
6. Dewax dried sections and pass to 90 per cent alcohol through usual reagents.
7. Stain until nuclei darken (1 to 3 minutes) in Ehrlich's acid alum hematoxylin.
8. Take each slide individually and flush surface with 96 per cent alcohol from a pipette. When sufficient stain has been removed, place slide in a saturated solution of lithium carbonate in 70 per cent alcohol until blue.
9. Dehydrate, clear, and mount in balsam.

Index

Acetic acid, contribution to nuclear fixation, 7
  for differentiating Chamberlain's stain, 108
  in, Allen's fixative, 11
    Berlese's mountant, 36
    Bouin's fixative, 10
    Carnoy and Lebrun's fixative, 10
    Ehrlich's hematoxylin, 18
    Gates' fixative, 9
    Gilson's fixative, 10
    Heidenhain's fixative, 9
    Johansen's Fast Green FCF, 28
    Kolmer's fixative, 11
    Lavdowsky's fixative, 8
    Navashin's fixative, 9
    Zenker's fixative, 8
Acetone, for dehydration, 44
  in Gray and Wess' mountant, 35
Acid fuchsin in, Chamberlain's stain, 106
  Mallory's stain, 25
  van Gieson's stain, 23
Adhesive, Gravis', for frozen sections, 74
  Mayer's, for paraffin sections, 67
Agar in, Gravis' adhesive, 74
  temporary wholemounts, 39
Alcohol, dehydrating agents for, 34
  for, Carnoy and Lebrun's fixative, 10
    Gilson's fixative, 10
    preserving plant tissues, 106
    storing wax blocks, 60
  recommended series of, 30, 55, 70
  substitutes for, in dehydration, 31
Allen's fixative, formula, 11
  for rat testis, 120
Alum (*see* Ammonium, Iron, etc.)
Ameba, 42
Ammonium acetate, 12
Ammonium alum, 18
Ammonium oxalate, 22
Amphioxus, recommended fixation, 128
  staining sections in Mallory's stain, 129
  treatment after Bouin fixation, 128
Anderson's embedding medium, 72, 73
Annelida, 43
Antiformin, 104
Aristolachia, 117–119
Arsenous oxide, 35
Arthropods, collection by Berlese funnel, 81
  making wholemounts of, 43

Bacteria, acid fast, 104
  Gram positive, 102
Bacterial smears, preparation of,
      100, 102, 104
  staining with, crystal violet, 100, 102
    magenta, 104
    methylene blue, 105
    safranin, 103
Balsam, Canada (*see* Canada balsam)
Bark, collecting invertebrates from, 81
Basic fuchsin (*see* Magenta)
Bayberry wax, 56
Beeswax, 56
Beetles, 82
Benzamine hydrochloride, 14
Benzene, for clearing tissues, 32, 111, 125
  unsuitability for clearing wholemounts, 45
Berlese's funnel, 81
Berlese's mounting medium, formula, 36
  cleaning from wholemounts, 75
  mounting mites in, 81
Bleaching, 97
Blood smears, mountant for, 36
  preparation of, 49
  stain for, 27
Blueing hematoxylin stains, 17, 93, 133
Bone, softening before sectioning, 13
  stains for, 27, 29
Borax in, alcohol as preservative, 29
  Grenacher's stain, 19
  stain for bone, 29
Bouin's fixative, formula, 10
  softening muscular tissues fixed in, 128
  washing from tissues, 12
Box, paper, for embedding, 59
Bryozoa, collecting and mounting, 84
  fixation of, 43
  narcotization of, 14, 43, 84
Butyl alcohol, 55

Calcium sulfate, 34
Camphor, 37
Canada balsam, alteration of stains by, 124
  cleaning from wholemounts, 45
  discussion of properties of, 37
  finishing slides mounted in, 47, 75
  making wholemounts in, 45
  mounting sections in, 71
  natural, for wholemounts, 88
  with salicylic acid, 130
Carazzi's hematoxylin, for chick embryo, 89
  formula for, 18
  in Patay's stain, 26
Carbolmagenta, 22
Carbolxylene, 32
Carmine, as nuclear stain, 19
  Grenacher's alcoholic, 19
  Mayer's alum, 20
  method for liver fluke, 95
  method for Pectinatella, 84
Carnoy and Lebrun's fixative, 10
Carrot, use in hand sectioning, 53, 106
Cartilage, stains for, 26, 27, 29
Cedar oil, 55
Celestin blue B, as nuclear stain, 21
  method for intestine, 110
  method for tongue, 125
Cellosolve, as dehydrating agent, 31
  as stain solvent, 28
  in Hanley's narcotic, 14

Cells, for thick wholemounts, 41
Celluloid, 41
Chamberlain's stain, 106, 107
Chick embryo, clearing, 93
  differentiating after staining, 93
  embedding, 58
  fitting paper support to, 91
  fixative for, 92
  removal from egg, 89
  removal of vitelline membrane from, 90
  sections of, 131
  staining, 92
  storage of, 92
  wholemount of, 89
Chitin, softening, 14
Chloral hydrate, as narcotic, 43
  in, Berlese's mountant, 36
    Gray's narcotic, 14
    Jurray's chitin softener, 14
Chloroform, as narcotic, 43
  for clearing, 32
  in Carnoy and Lebrun's fixative, 10
Chromic acid, as immobilizing agent, 6
  in, Allen's fixative, 11
    Gates' fixative, 9
    lo Bianco's fixative, 9
    Navashin's fixative, 9
Chromosomes, 122
Cladocera, 82
Cleaning, coverslips, 115
  prepared slides, 75
  slides, before use, 49, 113
Clearance angle, 62
Clearing, before embedding, 55
  discussion of, 30, 32
  in humid weather, 33
  special techniques, 33
Clip, for coverslips, 46
Clove oil, as stain solvent, 28
  for clearing, 32
Cocaine, 43
Cochineal, 15
Coelenterata, 14, 42
Collembola, 82
Connective tissue, 26, 27
Conn's crystal violet, 22
Contrast stains, 23
Coplin jar; 5
Copper nitrate, 11
Copper oleate, 37
Copper sulfate, 34, 111
Cork, 53
Coverslips, applying to sections, 71
  applying to wholemounts, 45, 88
  cleaning, 115
  clip for, 46
  methods of supporting, 41
  types of, 3
  use in flattening specimens, 85
"Craf" fixatives, 9
Crystal violet, for staining bacteria, 100, 102
  formula for Lillie's, 22
Crystals in sections, 12
Cutting facet, 62
Cytoplasmic detail, preservation of, 7

Decalcification, 12, 13
Dehydration, before paraffin embedding, 55
  discussion, 30
  Johansen's mixture for, 28
  special jar for, 111
  technique of, 33, 44
Dextrin, 72
Dextrose, 36
Differentiating, chick embryos, 93
  chromosomes, 123
  Ehrlich's hematoxylin, 133
  Grenacher's borax carmine, 87
  iron hematoxylin, 122
  liver fluke, 97
  plant skeletal tissues, 108
  tubercle bacilli, 105
Dioxane, for dehydration, 31, 55
  in mountant, 37
Dobell's iron hematoxylin, 16

Earthworm, monocystis in, 98
Egg albumen, as ribbon adhesive, 67
  in mounting protozoa, 42
Eggs, heavily yolked, staining nuclei in, 21
  staining cell walls in, 24
Ehrlich's hematoxylin, differentiating, 18, 133
  formula for, 18
  staining chick embryo with, 131
Elderberry pith, 53
Embedding, containers for, 58
  media, Anderson's, 72
    choice of, 56
    Hance's, 56
    Maxwell's, 56
  ovens, 57, 126
  paper box for, 59
  technique for, 57–60
Embryo, chick, wholemount of, 89
Embryological material, preservation,
Embryological watchglass, 4
Embryos, staining skeleton in, 29
Eosin, as contrast stain, 23
  compound with methylene blue, 27
  ethyl, 23
  staining intestine with, 110
Eosin Y, 23
Essential oils, dehydrating, 34
  for clearing, 32, 55
Ether (*see* Ethyl ether)
Ethyl alcohol (*see* Alcohol)
Ethyl cellosolve, 14

Ethyl eosin, 23
Ethyl ether, as narcotic, 43
  in Petrunkewitsch's fixative, 12
Eucalyptol, 37
Euparal, 36
  green, 37
External form, preservation of, 6
Eyes, 11

Farrants' medium, cleaning from mounts, 75
  formula for, 35
Fast Green FCF, as contrast stain, 23
  in Johansen's stain, 28, 118
Fats, handling sections of, 74
  section technique for, 71
  stains for, 29
Fish fry, 29
Fish scales, 40
Fixation of, Ameba, 42
  Amphioxus, 128
  Annelida, 43
  Aristolachia, 117
  Bryozoa, 43
  chick embryos, 92
  coelenterata, 43
  eyes, 11
  hard-shelled material, 10
  liver fluke, 95
  nervous tissue, 11
  Pectinatella, 85
  plant tissues, 8
  Polychaetae, 43
  Protozoa, 42
  rat testes, 120
  smears, 50
Fixative, formula for, Allen's, 11
    Bouin's, 10
    Carnoy and Lebrun's, 10
    Gates', 9
    Gilson's, 10
    Heidenhain's, 9
    Helly's, 8
    Kolmer's, 11
    Lavdowsky's, 8
    lo Bianco's, 9
    Navashin's, 9
    Petrunkewitsch's, 11
    Zenker's, 8
  functions of, 6
  removal of, 12, 110
  zoological general purpose, 10, 12
Flaming bacterial smears, 100
Flattening, chick embryo, 90
  liver fluke, 95
  specimens for wholemounts, 85
Flatworms, 20
Fleas, 82
Flotation method of clearing, 33
Formaldehyde, contribution to preservation of shape, 7
  in, Allen's fixative, 10
    Bouin's fixative, 10
    Heidenhain's fixative, 9
    Helly's fixative, 8
    Johansen's safranin, 22
    Kolmer's fixative, 11
    McNamara, Murphy and Gore's decalcifier, 13
    Navashin's fixative, 9
  preserving Bryozoa with, 43, 85
"Formol-Zenker" (*see* Heidenhain's fixative)
Freehand sections, fixing tissues for, 53
  methods of supporting, 53
  microtome for, 52
  staining and mounting, 53
Frog, killing for histological purposes, 110
  larva, staining nuclei in, 21
  section of intestine, 110
Frozen sections, adhesive for, 74
  cutting without embedding, 73
  embedding tissues before cutting, 73
  flattening, 74
  lacquer for, 74
  method of freezing, 73
  microtome for, 72
  mounting and staining, 74
  purpose of, 71
Fuchsin, basic (*see* Magenta)
Fungi, parasitic, 29
Gilson's fixative, for chick embryo, 92
  for liver fluke, 95
  formula of, 10
Glass cells, 41
Glycerine-alcohol, for storing wax blocks, 60
Glycerine in, gum mountants, 35
  Mayer's adhesive, 67
Gold size, 41
Gram positive bacteria, 102
Gram's iodine, formula for, 12
  in staining bacteria, 102
Gravis' adhesive, 74
Gray and Wess' mountant, cleaning from wholemounts, 75
  formula for, 35
Gray's, double contrast stain, 24
  narcotic, 14
Grenacher's alcoholic borax carmine, for invertebrate larvae, 44
  for Pectinatella, 84
  formula of, 19
Grinding microtome knives, 62
Gum acacia, 35, 36
Gum arabic (*see* Gum acacia)
Gum mastic, 74
Gum sandarac, 37

Hairs, 40
Half-ground microtome knife, 62
Hance's rubber paraffin, 56
Hanley's narcotic, 14
Hard-shelled material, fixation of, 10
Hard specimens, softening before sectioning, 13
Hardwood block holders, 65
Haug's decalcifier, 13
Heat, as immobilizing agent, 6
  for, fixing bacteria, 100
    flattening sections, 67
    rapid hematoxylin staining, 122
  in staining tubercle bacilli, 105
Heidenhain's, fixative, 9
  stain, 120
Helly's fixative, 8
Hemalum, 18
Hematein, 15
  for mordant staining, 16
Hematoxylin, blueing, 17, 93
  Carazzi's, 18, 26, 89
  contrast stains for, 23, 24, 26
  direct staining with, 17
  Dobell's, 15
  Ehrlich's, 18
  Heidenhain's, 120
  indirect staining with, 18
  mordant staining with, 15
  Régaud's, 16
Herbarium specimens, preparation for sectioning, 106
Holders, for wax blocks, 64, 65
Hollow ground microtome knife, 62
Hot plate, 68
Hucker's crystal violet, 22
Hydrocarbons for clearing, 32
  dehydrating, 34
Hydrochloric acid for differentiating, hematoxylin, 16, 18, 93
  toluidine blue, 29
Hydrozoa, fixation of, 6
  narcotization of, 42

Immobilizing agents, 6
Incubation of chick eggs, 89
Index cards, for storing slides, 77
Insects, 14
Intestine, 110–115
Invertebrate larvae, 44
Invertebrates, collecting, 81
  fixation of, 9
  narcotization of, 14
Iodine, for washing out fixatives, 12
  Gram's, formula for, 12
  in Chamberlain's stain, 106, 108
  Lugol's, formula for, 12
Iron alum, as mordant for hematoxy
  in celestin blue B solution, 21
Iron alum—*(Continued)*
  preparation of solutions of, 121
Iron hematoxylin, differentiation of, 122
  Dobell's, formula for, 15
  for spermatogenesis, 120
  Heidenhain's, formula for, 120
  quick method of staining with, 122
  Régaud's, formula for, 16

Jar, special for dehydrating, 111
Johansen's, quadruple stain, 28, 118
  safranin, 22
Jurray's chitin softener, 14

Kolmer's fixative, 11

Labels, 47, 76
Lactic acid in, Gray and Wess' mountant, 35
  tissue reviver, 106
Lavdowsky's fixative, 8, 117
Leaf, 53
Leeches, 43
Lenoir's reagent, 12
Lens paper, 39
Light green, 26
Lignified tissues, 29, 108
Lillie's crystal violet, 22, 100
Lithium carbonate for, blueing hematoxylin, 123, 133
  removing picric stain, 12, 120
Liver, 58
Liver fluke, anesthetizing, 95
  bleaching, 97
  collecting, 95
  fixing, 95
  staining, 20, 96
lo Bianco's fixative, 9
Logwood, 15
Loop, bacterial, 100
Lugol's iodine, 12

McNamara, Murphy and Gore's decalcifier, 13
Magenta, 22
Magnesium sulfate, 14, 43
Mallory's stain, applied to Amphioxus, 129
  formula for, 25
  mercuric chloride, as mordant for, 128
  phosphotungstic acid in, 128
  salicylic balsam for, 130
Marine forms, 9, 14
Mastic (*see* Gum mastic)
Maxwell's embedding medium, 56
Mayer's albumen adhesive, 67
Mayer's carmalum, for staining,
    tissues before embedding, 131
    liver fluke, 95
  formula for, 20
Meiosis, 11

Menthol, as narcotic, 43
  for Bryozoa, 84
  for liver fluke, 95
  in Gray's narcotic, 14
Mercuric-acetic fixatives, 10, 43
Mercuric chloride, as mordant, 128
  causing crystals in sections, 12
  in, Gilson's fixative, 10
    Heidenhain's fixative, 9
    Helly's fixative, 8
    Lavdowsky's fixative, 8
    McNamara, Murphy and Gore's decalcifier, 13
    Zenker's fixative, 8
Metaphase figures, 123
Methyl alcohol for, dehydration, 44
  fixing smears, 51
Methyl blue, 25
Methyl cellosolve, 22
Methyl violet 2B, 28
Methylene blue, 105
Methylene blue-eosin, 27, 51
Microstomum, 43
Microtome, adjusting block on, 66
  choice of, 61
  for frozen sections, 72
  knife, 61
    action in cutting frozen sections, 72
    clearance angle, 62
    cutting angle, 66
    grinding, 62
    half ground, 62
    removing nicks from, 63
    setting, 62, 63
    sharpening, 61–63
    square ground, 62
    stropping, 64
  object holders for, 64, 65
  rotary, 61
  Schantz, 61
  Spencer, 61, 65
Mites, 81
Mohr and Wehrle's mountant, 37
Monocystis, 98
Mordant staining, 15
Moss, 81
Mountants (*see also* Canada balsam)
  Berlese's, 36
  Farrants', 35
  Gray and Wess', 35
  gum, 35, 36
  Mohr and Wehrle's, 37
  resinous, 41
Mounting wax blocks on microtome, 64
Muscle, clearing, 125
  fixing, 125, 128
  staining, 24, 26, 27

Narcotic, Gray's, 14
  Hanley's, 14
Narcotization, 14, 42
Narcotizing, Bryozoa, 84
  invertebrates, 42–44
  liver fluke, 95
Navashin's fixative, 9
Neelsen's stain, 104
Nereis, 43
Nervous tissue, 11
Neutral mountant, 36
Nitric acid for, differentiating tubercle bacilli, 105
  in, Gilson's fixative, 10
    Haug's decalcifier, 13
    McNamara, Murphy and Gore's decalcifier, 13
    Petrunkewitsch's fixative, 11
  softening Bouin fixed muscles, 128
Nuclear detail, preservation of, 7
Nuclei (*see also* Chromosomes)
  staining, 15
    celestin blue B, 21, 110, 125
    hematoxylin, 16–19, 120, 131
    safranin, 22
    with carmine, 19

Oil blue N, 29
Oil stone, 63
Oligochaetae, 43
Orange G, 25
Orange II, 24
Oribatidae, 81
Osmic acid, 6, 50, 51
Ostracoda, 82
Ovens, embedding, 57, 58
Oxalic acid, for bleaching, 97
  in Mallory's stain, 25

Paper, black, for section identification, 131
  box, for embedding, 59
  for supporting chick embryos, 91
  in fixing liver fluke, 95
Paraffin, clearing before embedding in, 55
  dehydrating before embedding in, 55
  impregnating tissues with, 57
  ribbons, attaching to slide, 67
    bubbles in, 69
    cutting, 66
    drying, 68, 69
    flattening, 67, 69
    frame for protecting, 66
    handling, 66, 67
    identifying sections in, 131
    joining sections to form, 132
    mounting, 67
    mounting individual sections from, 113
    removing plant sections from, 106
    removing wax from, 70
    squeezing to slide, 69

Paraffin—*(Continued)*
  sections, choice of embedding medium for, 56
    choice of fixative for, 54
    staining and mounting, 69
    technique of dehydrating, clearing and embedding, 56
Paraldehyde, 37
Paramecium, 39, 40
Parasitic fungi, 29
Parlodion, 74
Patay's stain, 26
Pectinatella, 84–88
Petroleum jelly, 39
Petrunkewitsch's fixative, for intestine, 110
  for rat tongue, 125
  formula for, 11
Phenol, as coupling agent, 32
  in, Farrants' mountant, 35
    Jurray's solution, 14
    Petrunkewitsch's fixative, 12
    tissue swelling solution, 106
Phenyl salicylate, 37
Phloroglucinol, 13
Phosphomolybdic acid, 26
Phosphotungstic acid, 25, 128
Picric acid, for differentiating hematoxylin, 16
  in, Allen's fixative, 11
    Bouin's fixative, 10
    dehydrating alcohols, 127
    Smith's stain, 24
    van Gieson's stain, 23
  removal from tissues, 12
Picro-fuchsin, van Gieson's, 23, 126
Picro-spirit blue, Smith's, 24
Pipette, 4
Pith, 106
Plant chromosomes, fixation, 9
Plant stem, 117
Plant tissues, choice of stain for wholemounts of, 44
  reagents for skeletonizing, 107
Plasma stains, 23
Plaster of Paris, 111
Platyhelminthes, 43
Polychaetae, 9, 43
Polyvinyl alcohol, 35
Ponceau 2R in, Gray's stain, 24
  Patay's stain, 26
Potassium alum, as stain diluent, 96
  in, Carazzi's hematoxylin, 18
    Mayer's carmine, 20
Potassium dichromate, contribution to preservation of shape, 7
  fixative properties of, 7
  in, Heidenhain's fixative, 9
    Helly's fixative, 8
    Kolmer's fixative, 11
    Lavdowsky's fixative, 8
    Zenker's fixative, 8
Potassium hydroxide, 82
Potassium hypochlorite for, hydrolyzing sputum, 104
  skeletonizing plant tissue, 107
Potassium permanganate, 97
Prothallium, 20
Protoplasm, 7
Protozoa, fixation of, 42
  smears of, 16
  staining with carmine, 20
Pseudoscorpions, 82
Pyroxylin, 74

Quadruple stain, Johansen's, 28

Radiant heat oven, 57, 126
Rat, 120
Régaud's hematoxylin, 16
Rhizopoda, 42
Root, 106
Root tip, 9
Rope, for collecting Bryozoa, 84
Rotary microtome, 61
Rotifera, 14
Rubber paraffin, Hance's, 56
Rubber roller, for flattening sections, 69

Safety razor blade, as microtome knife, 61
  for trimming blocks, 64
Safranin, as nuclear stain, 22
  for staining bacteria, 102
  in Johansen's stain, 28, 117–119
Salicylic acid balsam, 26, 130
Saline, 90, 99
Salol, 37
Schantz microtome, 61
Scouring powder, 49
Sea anemones, 14
Section lifter, 4
Section of, Amphioxus, 128
  Aristolachia, 117
  frog intestine, 110
  rat tongue, 125
  root, 106
  72-hr. chick embryo, 131
Section planes, 52
Sectioning herbarium specimens, 106
Sections, for diagnostic purposes, 71
  freehand (*see* Freehand sections)
  frozen (*see* Frozen sections)
  identifying side of slide carrying, 69
  joining to form ribbons, 132
  mounting in balsam, 71
  paraffin (*see* Paraffin ribbons, Paraffin sections)
Setting microtome knives, 62, 63
Sharpening microtome knives, 62, 63
Shrinking, control of, in fixation, 7
Skeleton, animal, 29
  plant, 106

Slide cabinets, 76, 77
Slides, cleaning, 49, 113
  finishing, 75, 76
  flattening specimens between, 85
  kinds of, 3
  storage of, 76
  with ground end for labeling, 76
Smears, bacterial, 100, 102, 104
  blood, 27, 49
  fixation of, 50, 98
  protozoan, 16, 98
  staining, 16, 51
  thick, 50
Smith's picro-spirit blue, 24
Sodium acetate, 22
Sodium carbonate, 93
Sodium hypochlorite for, hydrolyzing sputum, 104
  skeletonizing plant tissues, 107
Sodium salicylate, 67
Sodium sulfate, fixative properties of, 7
  in, Helly's fixative, 8
    Zenker's fixative, 8
Solvents, "universal," 31
Spencer microtome, 61
Spermatogenesis, 120
Spirit blue, 24
Sporozoa, 98
Sputum, 104
Square ground microtome knife, 62
Staining, bacteria, 100, 102, 104
  chromosomes, 122
  intestine, 114
  jars for, 5
  lignified tissues, 108
  plant skeletons, 106
  smears, 51
  solutions (*see* under inventor, ingredient or purpose)
  techniques, complex, for animal tissues, 25
    for plant tissues, 27
Steam, for fixing, 42, 50
Stender dish, 4
Stones, sharpening, 63
Stropping microtome knives, 64
Sudan IV, 29
Sulfuric acid, 21
Synthetic nuclear stains, 21
Syracuse watch glass, 4
Syrup, 72

Tap water, for blueing hematoxylin, 17, 93
Terpineol for clearing, 32
  chick embryos, 93
  frozen sections, 74
  wholemounts, 45, 87
Thysanura, 82
Tin cells, 41
Tissue blocks, staining before embedding, 20
Toluene, 32
Toluidine blue, 29
Tongue, 125
Transparency, preservation of, 7
Trichloroacetic acid in, Kolmer's fixative, 11
  McNamara, Murphy and Gore's decalcifier, 13
Triple stains, 25, 26
Tubercle bacilli, 104
Tubes, for handling small objects, 45, 86
Turbellaria, 43
Tyroglyphidae, 82

Uranyl acetate, 11
Urea, 11

van Gieson's picro-fuchsin, 23, 126, 127
van Wijhe's stain, 29
Vertebrate embryos, 44
Vials, 4, 5
Vortex, 43

Washing fixed tissues, 110
Watchglasses, 4, 58
Water immersion objectives, 123
Water stone, 63
Wax blocks, adjusting on microtome, 66
  mounting on holder, 64, 65
  storage of, 60
  trimming, 64
Wholemounts, applying coverslip to, 88
  choice of, clearing agent for, 45
    stain for, 44
  cleaning, 75
  cleaning and labeling, 47
  flattening specimens for, 85
  in gum media, 40
  of chick embryo, 89
  of liver fluke, 95
  of mite, 81
  of Pectinatella, 84
  of thick objects, 40
  temporary, 39
Wright's stain, 27

Xylene, 32

"Zenker-formol" (*see* Heidenhain's fixative)
Zenker's fixative, 8, 43
Ziehl-Neelsen's technique, 104
Ziehl's carbolmagenta, 22
Zimmerman's lacquer, 74